A NEW METHOD FOR MUTANT SELECTION IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

A new method for the selection of auxotrophic, antibiotic- and temperature-sensitive mutants in *Saccharomyces cerevisiae* is reported. The technique is based upon the observation that certain fatty acid auxotrophs of yeast die when deprived of fatty acid only under conditions supporting growth. When macromolecular synthesis is blocked, the fatty acid-starved cells survive. By appropriate manipulation of a fatty acid-requiring strain enrichment as great as 75-fold was achieved for certain classes of auxotrophic mutants. An enrichment of approximately 100-fold is possible for some antibiotic-sensitive mutants. Selection for temperature-sensitive mutants, however, resulted in less than a 2-fold increase in the frequency of such mutants, probably because of the heterogeneity of this mutant category. It is likely that only that fraction of temperature-sensitive mutations which rapidly and reversibly blocks macromolecular synthesis is selected by this technique.

HIGHLY effective techniques for the selection of various classes of mutants have been developed for bacteria. These techniques include penicillin enrichment (DAVIS 1948) and tritium suicide (CRONAN, RAY and VAGELOS 1970; REID 1971). Analogous methods of nystatin enrichment (SNOW 1966) and tritium suicide (LITTLEWOOD and DAVIES 1973) are available for yeast. However, additional methods for selection in yeast may be of value in the isolation of certain mutant classes. Thus, the yeast "kamikaze" mutant, which dies at 42° unless protein synthesis is blocked, was successfully used in the isolation of mutants sensitive to inhibitors of protein synthesis (LITTLEWOOD 1972).

In other fungi, such as *Neurospora* (LESTER and GROSS 1959), *Aspergillus* (PONTECORVO *et al.* 1953) and *Ustilago* (HOLLIDAY 1962; THOMAS 1972), the "unbalanced growth" exhibited by certain auxotrophic mutants has been successfully used in mutant selection. The conidia of inositol auxotrophs of *Neurospora* and *Ustilago* and biotin auxotrophs of *Aspergillus* were found to die if permitted to germinate in the absence of the auxotrophic requirement, but conidia having a second auxotrophic requirement preferentially survived.

Similarly, fatty acid-requiring mutants of yeast were recently reported to die if starved for fatty acid under otherwise growth-supporting conditions (HENRY 1973). If protein synthesis in these cells was prevented, either by cycloheximide or by starvation for lysine in a strain also auxotrophic for this amino acid, cell

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death was largely prevented. These results suggested that it might be possible to devise a method for the selection of a variety of mutant classes including certain types of temperature- and antibiotic-sensitive mutants as well as auxotrophs. This report presents a new general method for mutant enrichment in yeast based on these observations.

MATERIALS AND METHODS

Strains: The strain BW1 1C *a ole1 fas1* has been described elsewhere (HENRY 1973). This strain requires both saturated and unsaturated fatty acids for growth. The strain BL15 is the "kamikaze" strain referred to earlier (LITTLEWOOD 1972).

Chemicals: The detergents Tergitol NP40, Tween 40 and Tween 80 were obtained from Sigma Chemical Company, St. Louis, Mo. Ethyl methanesulfonate (EMS), cycloheximide, nystatin, and 5-fluorouracil were also obtained from Sigma Chemical Co. Ethidium bromide was supplied by Boots Pure Drug Co., Ltd.

Growth media: The media used are constituted as follows and will be referred to by the following abbreviations: *YEPD*: Difco yeast extract (1%), Difco bactopectone (2%) and D-glucose (2%). *YTT*: YEPD plus Tween 40 (1%) and Tween 80 (1%). *YTT* satisfies the fatty acid requirements of the strain BW1 1C. *COM*: a complete synthetic medium described elsewhere (HAWTHORNE and MORTIMER 1960) containing yeast nitrogen base without amino acids and D-glucose as well as adenine, uracil, L-arginine, L-histidine, L-leucine, L-lysine, L-methionine, L-threonine, and L-tryptophan. Whenever one or more of these supplements is omitted from the medium, it will be indicated as COM —; (for example, COM — methionine indicates methionine has been omitted). *MIN*: a synthetic medium containing Difco yeast nitrogen base without amino acids (0.67%) and D-glucose (2%).

In order for the synthetic media to support the growth of BW1 1C, fatty acid must be supplied. In agar plates this was always done by adding Tween as in *YTT*. This will be denoted throughout this report as + Tween; (for example; COM + Tween). In liquid culture, the fatty acid requirement was met by adding oleic acid (10^{-3} M) and myristic acid (10^{-3} M) solubilized with Tergitol NP40 (3%). Whenever the media referred to in this report contain these fatty acids, it will be noted as + fatty acids (for example; COM + fatty acids). However, Tween 80 (1%) + Tween 40 (1%) in liquid culture will satisfy the requirement and can be substituted. Tergitol NP40 in the absence of fatty acids was observed to accelerate cell death (HENRY 1973). Therefore, Tergitol NP40 (3%) was added to all fatty acid starvation media. Such media will be designated + Tergitol (for example; COM + Tergitol, *YEPD* + Tergitol).

Selection procedures: In order for selection to be successful, the number of revertants and petites must be minimal. To accomplish this, the strain should be cloned on *YTT* agar medium just prior to use and the clone or clones to be used should be tested for fatty acid requirement and respiratory sufficiency by appropriate replica plating.

The entire procedure including mutagenesis and selection requires 3 days for a single round of enrichment.

Day 1: Liquid *YTT* medium is inoculated with freshly tested BW1 1C cells. This culture is permitted to grow with agitation at 30° for at least 24 hours or until a stationary culture is achieved. It is not advisable to let this strain remain for an extended period of time in stationary phase.

Day 2: BW1 1C cells taken from the stationary culture are mutagenized with 3% EMS for 90 minutes at 25° following a standard protocol (LINDEGREN *et al.* 1965). This exposure to EMS gives approximately 30% survival. Following mutagenesis and inactivation of the EMS, the cells are washed twice and resuspended in liquid *YTT* medium at a density of approximately 2×10^6 cells/ml. This culture is allowed to grow at 25° with agitation overnight, or at least 12 hours. To assure that the cells are growing vigorously, the culture should not be used for enrichment until it has at least doubled in optical density.

Day 3: The procedure to be used at this stage will depend upon the type of mutant to be

selected. Three categories of mutants will be dealt with separately: auxotrophic, temperature-sensitive, and antibiotic-sensitive mutants.

1. *Auxotrophic mutants*: By choosing the appropriate selective medium, mutants with any of a variety of auxotrophic requirements can be selected. In this paper, selection of "total auxotrophs" implies the simultaneous enrichment of mutants having an auxotrophic requirement for any one of the following compounds: adenine, uracil, L-arginine, L-histidine, L-leucine, L-lysine, L-methionine, L-threonine and L-tryptophan. Others could have been selected for as well. Alternately, the selection of a "single class of auxotrophs" implies enrichment for mutants with an auxotrophic requirement for one of the above mentioned compounds without simultaneously enriching for auxotrophs in the others. The following is the protocol used for the enrichment of "total auxotrophs". The protocol for the selection of a "single class of auxotrophs" is identical to that described except that MIN is replaced at each step by COM minus the compound which is needed to satisfy the auxotrophic requirement of the mutant (for example; COM — methionine replaces MIN when methionine auxotrophs are specifically desired). The detergent supplements to the media remain unchanged.

For total auxotroph selection, the mutagenized cells, after overnight growth, are first washed three times with liquid MIN + fatty acids medium and resuspended at a density of 2×10^4 or 2×10^6 cells/ml in this medium. This culture is then incubated at 35° for three hours. During this incubation period, culture medium should be replaced at 1-hour intervals with fresh MIN + fatty acids. This procedure helps to prevent crossfeeding of potential mutants. The three-hour period of incubation in minimal medium prior to fatty acid starvation assures that most auxotrophic mutants have depleted cellular pools of the required substances prior to fatty acid starvation. At the end of three hours, the cells are washed three times with MIN + Tergitol and resuspended in MIN + Tergitol at the previous cell density and incubated again at 35°. Fatty acid starvation begins at this point. Again, the medium should be replaced at regular intervals with fresh MIN + Tergitol to minimize crossfeeding. Cell death begins approximately two hours following the removal of fatty acid. Maximum enrichment is accomplished in approximately 12 to 18 hours. When a second round of selection is desired, the cells are simply allowed to recover in liquid YTT for 12 hours and then subjected to the entire procedure again, starting at "day three".

At various times during selection, the surviving cells are plated onto YTT plates at appropriate cell densities and allowed to recover. When a culture density of 2×10^4 cells/ml is used during selection, it is necessary to concentrate the culture by centrifugation followed by resuspension in 1/10 the previous volume before plating. If this is not done, at least in the later stages of cell death, insufficient cells remain viable to give adequate plating densities. After colonies form, they are replica plated onto MIN + Tween and YTT plates. After two days in incubation, those colonies failing to grow on MIN + Tween plates are picked from the YTT replica and streaked onto fresh YTT. These strains are tested after several days of incubation by replica plating onto a series of "drop-out" plates (each plate containing the ingredients found in COM + Tween but omitting one of the nine compounds listed on Table 1). In addition, the colonies are replica plated onto MIN + Tween, to verify auxotrophy, and onto COM + Tween, to identify auxotrophs not belonging to the nine classes listed on Table 1. After two days of incubation, the specific auxotrophic requirement of each strain is determined by comparison of the "drop-out" plates.

2. *Temperature-sensitive mutants*: The permissive and restrictive temperatures chosen for these experiments were 25° and 35° respectively. The recovered, mutagenized cells are incubated at 35° in YTT liquid medium at a density of 2×10^6 cells/ml. After 30 minutes the culture is subjected to centrifugation in a table-top centrifuge which is prewarmed to 35°. The cells are washed three times in YEPD + Tergitol liquid medium which is prewarmed to 35° and finally suspended in this medium at a density of 2×10^6 cells/ml. The culture is incubated with agitation at 35°, and, at various times, plated onto YTT plates and allowed to recover at 25°.

3. *Antibiotic-sensitive mutants*: Yeast are impermeable to certain antibiotics which would otherwise inhibit their growth. One such antibiotic is α -amanitin, the toxin isolated from mushrooms that has been shown to be a specific inhibitor of the DNA-dependent RNA polymerase II of eukaryotes (FIUME and WIELAND 1970, a review). It would be of value to isolate a mutant

of yeast permeable to α -amanitin in order to study its effect on RNA polymerase. The selection for such a mutant follows the procedure described for the selection of temperature-sensitive mutants, except α -amanitin (50 $\mu\text{g}/\text{ml}$) is added to the final suspension of the cells in YEPD + Tergitol liquid medium before incubation and the surviving cells are grown on YTT agar plates at 30°. In order to measure the potential for enrichment of an antibiotic-sensitive mutant, cycloheximide (100 $\mu\text{g}/\text{ml}$) is added to a separate, second suspension of cells and the percent survival in that culture is compared to the percent survival of cells in a third culture, to which no antibiotic is added.

Surviving colonies from the culture treated with α -amanitin are replica plated onto two YTT agar plates, one of which contains α -amanitin (50 $\mu\text{g}/\text{ml}$), and incubated at 30°. Colonies whose growth appears to be inhibited by α -amanitin are picked from the plate not containing the antibiotic and retested for sensitivity to α -amanitin.

RESULTS

The effectiveness of "fatty acid-less death" in the selection of auxotrophic, temperature-sensitive and antibiotic-sensitive mutants was tested. Since selection for each of the three categories of mutants requires different manipulations of the fatty acid-requiring strain, each will be considered separately. As a general first step, however, the survival during starvation of mutagenized fatty acid-requiring cells was compared to unmutagenized cells (Figure 1). Clearly, muta-

FIGURE 1.—Survival of mutagenized ($\bullet$) and unmutagenized ($\blacktriangle$) cells of BW1 1C starved for fatty acid in synthetic minimal medium with 3% Tergitol NP40 (MIN + Tergitol). Also illustrated is the increase in the % of methionine auxotrophs ($\blacksquare$) in a mutagenized cell culture prestarved for methionine and starved for fatty acid in COM — methionine + Tergitol (see MATERIALS AND METHODS for conditions and media). Arrow indicates the start of fatty acid starvation.

genized cell populations die significantly more slowly than unmutagenized cells and this is expected to affect potential selection.

Selection of auxotrophic mutants: nine types of auxotrophs (listed on Table 1), each of which is readily obtainable following mutagenesis with EMS, were chosen to test the selective potential of "fatty acid-less death." For these nine classes of auxotrophs it is possible to obtain, with considerable accuracy, the frequency of occurrence before and after selection. Real enrichment can, therefore, be calculated. In the case of rare mutants it would be very difficult to measure real enrichment because of the problem of estimating starting frequencies. The increase in frequency achieved for these nine auxotrophic mutant categories should, however, serve as a measure of the potential effectiveness of this method when applied to mutant classes for which enrichment cannot be calculated. In addition, the comparison of enrichment achieved in each of the nine categories should demonstrate whether selection is equally effective for different types of mutants.

Figure 1 illustrates the increase in the frequency of methionine auxotrophs during the course of fatty acid starvation. The increase in methionine auxotrophy is greater during the first 12 hours and reaches a plateau by 18 hours. Consequently, in the selection of auxotrophic mutants, all cultures were starved for fatty acid for 18 hours.

After a single round of selection at a cell density of 2×10^6 cells/ml, the percentage of total auxotrophic mutants among surviving cells was 38.0% compared to 6.7% at the start of the experiment (Table 1). After two rounds of selection 64.1% of the survivors were auxotrophic.

Characterization of the surviving cells clearly demonstrates that enrichment was not equally successful for all nine classes of auxotrophic mutants. Methionine auxotrophs, for example, were enriched 21.1-fold in one round of selection while the frequency of adenine, arginine, histidine, threonine and uracil requirers was not significantly increased (Table 1). The remaining three classes of auxotrophic mutants show enrichments ranging from 10.5- to 4.3-fold. In addition, auxotroph classes other than those tested were present at a frequency of 2.9% at the start of the experiment. As a general class, these uncharacterized mutants increased in frequency by 4.3-fold after one round of selection (Table 1).

Greater enrichment was accomplished by allowing the cells surviving after the initial round of fatty acid starvation to recover overnight in the presence of fatty acid and then subjecting them to a second round of selection. As indicated in Table 1, the second round of selection resulted in 2- to 4-fold increase in the percentage of all auxotroph classes except the uncharacterized mutants. The second round of selection was especially effective for lysine auxotrophs. In general, the second round of selection was not as successful as the first round in increasing the frequency of auxotrophic mutants. This diminished effectiveness may result from the presence of revertants not requiring fatty acids.

Increased enrichment for a single class of auxotrophs was obtained when the culture was prestarved for that requirement alone while being supplied with a

TABLE 1
Selection of auxotrophic mutants

Auxotroph class	% at start of selection	% after one round of selection for total auxotrophs	enrichment	% after two rounds of selection for total auxotrophs	enrichment	% after one round of selection for a single auxotrophs class	enrichment	% after two rounds of selection for a single auxotrophs class	enrichment	% after one round of selection for a single auxotrophs class	enrichment	% after two rounds of selection for a single auxotrophs class	enrichment
Culture density (cells/ml) during selection		2×10^6		2×10^6		2×10^6		2×10^6		2×10^6		2×10^6	
TOTAL AUXOTROPHS	6.7	38.0	5.7	64.2	9.6	—	—	—	—	—	—	59.3	8.9
adenine	0.8	0.8	1.0	1.4	1.8	—	—	—	—	—	—	3.7	4.6
arginine	0.5	0.7	1.4	1.8	3.6	—	—	—	—	—	—	5.7	11.4
histidine	0.4	0.4	1.0	0.7	1.8	—	—	—	—	—	—	1.1	2.8
leucine	0.2	2.1	10.5	3.9	19.5	—	—	—	—	—	—	5.1	26.0
lysine	0.3	2.5	8.3	9.9	33.0	7.5	25.0	—	—	—	—	3.7	12.3
methionine	0.8	16.9	21.1	35.8	44.8	30.5	38.1	60.2	75.2	—	—	22.9	28.6
threonine	0.2	0.3	1.5	—	—	—	—	—	—	—	—	1.3	6.5
tryptophan	0.3	1.4	4.7	2.2	7.3	—	—	—	—	—	—	1.4	4.7
uracil	0.3	0.3	1.0	0.4	1.33	—	—	—	—	—	—	0.7	2.3
all others*	2.9	12.6	4.3	8.1	2.8	—	—	—	—	—	—	13.6	4.7
Number of colonies screened	3610	2070		879		681 (lys) 793 (met)		1035				2496	

Selection conditions for total auxotrophs and for single auxotroph classes are outlined in MATERIALS AND METHODS.

% equals $\frac{\text{no. of auxotrophic colonies}}{\text{no. of total colonies}} \times 100$; enrichment equals $\frac{\% \text{ after selection}}{\% \text{ at start of selection}}$.

* Category "all others" includes all auxotrophs which did not fall into the nine classes or which demonstrated more than one requirement.

large number of other potential requirements. This is illustrated in Table 1 for lysine and methionine auxotrophs. Lysine auxotrophs increased 25.0-fold during this method of selection as compared to 8.3-fold following selection for "total auxotrophs". The enhanced enrichment is presumably due to the elimination of other classes of auxotrophs, especially the methionine requirers.

Certain types of mutants, such as those requiring adenine, failed to increase in frequency during selection for "total auxotrophs" at a culture density of 2×10^6 cell/ml. In order to ascertain whether the lack of enrichment was due to failure of the specific auxotrophy to confer protection during fatty acid starvation, three strains were derived from cells which had survived one round of fatty acid starvation at a culture density of 2×10^6 cells/ml. One strain required adenine, one was auxotrophic for arginine and the third was a methionine auxotroph. The results diagrammed in Figure 2 show that all strains are protected during fatty acid starvation by prior starvation for the additional auxotrophic requirement. The important difference between such experiments and those involved in general selection is that here all cells are auxotrophic for the requirement being tested, eliminating the opportunity for cross feeding. It is interesting to note that the adenine and the arginine auxotrophs used in the above experiment died substantially slower even when supplemented with their respective requirements (Figure 2) than did the general population of mutagenized cells (Figure 1). Both of these strains were found to be petite, suggesting that their survival during the initial enrichment experiment may have been only partly related to their auxotrophy. The methionine auxotroph, in contrast, was not petite and in the presence of L-methionine, underwent cell death at a rate comparable to the mutagenized cells illustrated in Figure 1.

To test the possibility that failure to select certain classes of auxotrophic mutants was due to cross feeding, the selection for "total auxotrophs" was carried out at a cell density of 2×10^4 cells/ml. At this lower cell density, the potential for cross feeding should be reduced. The results are shown on Table 1. The frequency of total auxotrophs increased over that detected at higher cell concentrations and the increased enrichment was reflected in all classes of auxotrophs, including adenine and L-arginine.

Selection of temperature-sensitive mutants: It is expected that only those temperature-sensitive mutations which have the effect of rapidly stopping macromolecular synthesis without affecting cell viability will be selected by fatty acid starvation. It is unclear what fraction of the total population of temperature-sensitive mutants this class represents.

The frequency of total temperature-sensitive mutants was increased approximately 1.8 times in the first 8 hours and diminished somewhat by 18 hours to approximately 1.5 times the starting frequency (Table 2). After eight hours of fatty acid starvation approximately 97% of the starting cells were dead (Figure 1). If all temperature-sensitive mutations conferred complete protection against cell death, the frequency of temperature-sensitive mutants would be expected to rise well over 50% by eight hours of fatty acid starvation. Since this is not the case, it is probable that only a small fraction of the initial temperature-sensitive

FIGURE 2.—Survival during fatty acid starvation of protected and unprotected auxotrophic cells. Each auxotroph was derived from BW1 1C in the course of a selection experiment; A. adenine auxotroph; (○) prestarved for adenine; starved for fatty acid in COM — adenine + Tergitol; (△) supplemented with adenine during fatty acid starvation. B. arginine auxotroph; (□) prestarved for arginine, starved for fatty acid in COM — arginine + Tergitol; (■) supplemented with arginine during fatty acid starvation. C. methionine auxotroph; (●) prestarved for methionine; starved for fatty acid in COM — methionine + Tergitol medium. (▲) supplemented with methionine during fatty acid starvation (see MATERIALS AND METHODS for conditions and media). Arrow indicates the start of fatty acid starvation in each case.

mutations are able to protect the cells during fatty acid starvation. Without further characterization of these mutants, it is impossible to calculate the real enrichment being achieved but it can be estimated from the results obtained with the auxotrophic mutants and from the results of antibiotic experiments which follow.

Selection of antibiotic-sensitive mutants: It should be possible to use the technique of fatty acid starvation in the selection of mutants possessing increased

TABLE 2

Selection of temperature-sensitive mutants

% <i>ts</i> mutants at start of selection	% after 8 hrs of selection	enrich- ment	% <i>ts</i> mutants after 18 hrs of selection	enrich- ment
5.1	9.3	1.8	7.8	1.5
2384	1509		1821	no. of colonies screened

permeability toward antibiotics capable of inhibiting macromolecular synthesis. Since the initial frequency of occurrence of such a mutant may be immeasurably low, it is impossible to directly estimate the selectivity of this technique in isolating antibiotic-sensitive mutants. However, the protection afforded by cycloheximide to a culture undergoing fatty acid starvation should be a good measure of selectivity since cycloheximide rapidly inhibits macromolecular synthesis in yeast. Figure 3 illustrates that cell death as a result of fatty acid starvation is partially alleviated by cycloheximide. However, mutagenized cells treated with cycloheximide during "fatty acid-less" death do not retain viability as well as

FIGURE 3.—Effect of cycloheximide on death caused by fatty acid starvation. BW1 1C was starved for fatty acid as described under MATERIALS AND METHODS in the presence (●) and absence (○) of cycloheximide (100 μ g/ml). After 8 hrs the cells were resuspended in YTT liquid medium and allowed to recover at 25°. Cell viability was determined by plating onto YTT-agar plates. The potential for enrichment (■) was calculated by dividing the % survival observed in the presence of cycloheximide by the % survival observed in the absence of cycloheximide.

unmutagenized cells and the mutagenized cells die less rapidly than unmutagenized cells when not protected (Figure 1). Consequently, one round of selection should increase the abundance of antibiotic-sensitive cells by 8–9-fold (Figure 3).

However, fatty acid-starved cells recover growth very slowly and may require more than 10 hours to resume any growth (HENRY 1973). Thus it is very interesting to note that cells protected by antibiotic recover more rapidly than unprotected cells, thus increasing the potential for enrichment for antibiotic-sensitive cells to 90–100-fold (Figure 3). Of course, this estimate of selectivity based on the action of cycloheximide assumes that other antibiotics which inhibit macromolecular synthesis provide similar protection and are as rapidly reversible.

This technique was used in an attempt to isolate mutants sensitive to α -amanitin as described under MATERIALS AND METHODS. Using cycloheximide as a measure of selectivity, a 100-fold enrichment was achieved. Eight hundred of the surviving colonies were examined for sensitivity to α -amanitin (50 μ g/ml) and none were found to be sensitive. If our estimate of enrichment is accurate, it is concluded that less than one in 80,000 cells shows the phenotype of sensitivity to α -amanitin after EMS mutagenesis.

DISCUSSION

The results demonstrate that certain classes of mutants can be effectively selected by manipulation of a fatty acid-requiring strain. The enrichment is the result of the protection conferred on fatty acid-starved cells by mutations affecting macromolecular synthesis. By this technique, certain auxotrophic mutants can be increased in frequency as much as 75-fold.

The failure of this technique to select other classes of auxotrophic mutants cannot be ascribed to the inherent failure of the particular auxotrophic requirement to protect the cell during "fatty acid-less-death". Rather, the results suggest that the failure may be due in part to cross feeding in the mixed population of mutagenized cells. This can be avoided to some extent by using more dilute cell suspensions. The problem of cross feeding is not likely to be limited to this particular technique of mutant enrichment, but will interfere with any selection scheme for auxotrophic mutants that depends upon selective killing of growing cells, including the nystatin technique (SNOW 1966) and any method utilizing the "kamikaze" mutant (LITTLEWOOD 1972).

Even at the lower cell density, however, selection does not reach the effectiveness which might be predicted from the survival curves in Figure 1. If the protection conferred by a mutation were totally effective, increases in mutant frequency of well over 100-fold should be achieved in one round of cell death. Clearly, this protection conferred by auxotrophic mutants is not complete. This is demonstrated by the survival curves of the protected cells in the homogeneous populations illustrated in Figure 2. It is possible, even in the protected cells in the homogeneous populations illustrated in Figure 2, that macromolecular synthesis is not completely blocked. Residual synthesis might result from utilization

of the required substance derived from degradation of existing macromolecules or from undepleted pools. Some auxotrophic mutants, for example, may require more than the three-hour starvation period used in these experiments to eliminate cellular pools of the required substances. This may explain the failure to obtain substantial enrichment of uracil- and histidine-requiring mutants even at the lower cell densities. Furthermore, no auxotroph is completely viable during extended starvation even though amino acid, purine and pyrimidine requirers are more viable during starvation than are fatty acid requirers (HENRY 1973). Since these are properties of the individual auxotrophic mutant or class of auxotroph which is being selected, some of these considerations may well explain the differences in effective enrichment of different classes of mutants.

One of the major drawbacks of fatty acid starvation as a selective technique is the necessity to cross out the fatty acid markers from the newly isolated mutants. This, of course, is also a problem with the "kamikaze" technique and the use of "inositol-less" death in other fungi. The difficulties, however, can be minimized by crossing the fatty acid-requiring strain BW1 1C α which contains the new mutation to an α (wild type for fatty acid requirement), sporulating and preparing random spores which are plated out on YEPD (no tween). Thus, only spores free of the fatty acid requirement will germinate and 50% of these should contain the newly isolated mutation.

Similar considerations apply to the selection of temperature-sensitive mutants. Not all temperature-sensitive mutants are viable at the restrictive temperature. We have preliminary results which suggest that approximately 50% of all temperature-sensitive mutants show substantial viability loss (without fatty acid starvation) at the restrictive temperature over the time period required for this selection technique. Of the remaining 50%, perhaps only a fraction shut off macromolecular synthesis rapidly enough to confer protection. It is to be expected that for these mutants, the degree of selection should be similar to that estimated by the cycloheximide technique. Therefore, this enrichment should be quite specific for mutations rapidly and reversibly affecting macromolecular synthesis.

Fatty acid starvation should prove effective in the selection of antibiotic-sensitive mutants. As judged by the ability of cycloheximide to prevent fatty acid-less death, an enrichment of 90–100-fold is routinely possible. However, an α -amanitin-sensitive mutant was not obtained using this technique. Unfortunately, the search was complicated by the relative scarcity and cost of α -amanitin.

It was of interest to compare fatty acid starvation to other techniques which are available for the selection of antibiotic-sensitive mutants. Using the protection afforded by cycloheximide as a measure, the selectivity of the following techniques was estimated: death of "kamikaze" at the non-permissive temperature (LITTLEWOOD 1972), death caused by nystatin (SNOW 1966) and petite formation induced by 5-fluorouracil (WHITTAKER, HAMMOND and LUHA 1972) or ethidium bromide (HOLLENBERG and BORST 1971; WHITTAKER, HAMMOND and LUHA 1972). In our hands, cycloheximide was only slightly effective in preventing the death of mutagenized cells incubated in the presence of nystatin and

in preventing either 5-fluorouracil or ethidium bromide from causing petite formation. Furthermore, the effects were not always reproducible. In contrast, the use of the "kamikaze" mutant was shown to be a potentially powerful tool in the selection of antibiotic-sensitive mutants. One round of this technique was found to have the potential for enriching antibiotic-sensitive cells by 100-fold. Thus, as a tool in the selection for antibiotic-sensitive strains, the use of drugs proved more laborious and less effective than the use of characterized conditional lethal mutants such as "kamikaze" and the fatty acid auxotroph described in this paper.

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