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Factors Affecting the Survival of Auxotrophs and Prototrophs of *Saccharomyces cerevisiae* in Mixed Populations

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ABSTRACT

MOAT, ALBERT G. (Hahnemann Medical College, Philadelphia, Pa.), ISABEL J. BARNES, AND ELEANOR H. McCURLEY. Factors affecting the survival of auxotrophs and prototrophs of *Saccharomyces cerevisiae* in mixed populations. J. Bacteriol. 92:297–301. 1966.—The conditions under which the number of yeast prototrophs, as well as respiration-deficient mutants, could be materially decreased, while allowing the survival of auxotrophic mutants in recoverable numbers, have been investigated in detail. Neither the use of carbohydrates other than glucose to prevent development of respiration-deficient mutants, nor treatment with acriflavine to render all surviving wild types respiration-deficient, provided a selective advantage for the auxotrophs. Increased concentrations of the antifungal agents amphotericin B or endomycin, while reducing the number of respiration-deficient mutants, did not significantly increase the final mutant-wild type ratio. A more soluble form of amphotericin B (Fungizone), when used under carefully defined physiological conditions, produced a significant reduction in the number of surviving prototrophs relative to the surviving auxotrophs, without development of respiration-deficient mutants.

A previous report from our laboratory (4) provided evidence that several antifungal agents, particularly amphotericin B and endomycin, are effective in reducing the number of yeast prototrophs in a mixed population of prototrophs and auxotrophs. However, the efficacy of these antibiotics as selective agents was limited by the survival of relatively large numbers of respiration-deficient mutants. The presence of these mutants made it necessary to add another step to the procedure to identify them, and to use greater dilutions for the final plating.

Several approaches were taken in attempting to solve these problems and to improve further the selectivity of procedures employed to eliminate prototrophs. Increased concentrations of amphotericin B and endomycin, coupled with plating on membrane (Millipore) filters, were employed to prevent survival of the respiration-deficient mutants. The use of carbon sources which would not permit growth of the respiration-deficient mutants, and preferential conversion of prototrophs, but not auxotrophs, to the respiration-deficient state, were also investigated. Fungizone (amphotericin B solubilized in deoxycholate and phosphate buffer) was studied to determine the physiological conditions under which it could most effectively serve as a selective agent for auxotroph isolation.

MATERIALS AND METHODS

The mixed population of yeast mutants and wild types utilized for these studies consisted of an adenine-requiring haploid mutant (*Saccharomyces cerevisiae* 173/1a), which was readily distinguishable from its parent prototroph (*S. cerevisiae* 174/1d) by virtue of the production of a pink pigment (1, 8) resulting from the accumulation of aminoimidazole ribonucleotide or its aglycone (2, 5).

Respiration-deficient mutants were identified by the triphenyltetrazolium chloride (TTC) overlay technique of Ogur et al. (6), since small colony size has not been found to be a reliable index of respiratory incompetency.

The medium employed for all experiments requiring a medium of known composition was that of Snell et al. (7) modified by eliminating zinc and copper salts and potassium iodide, which were found to be unnecessary for the growth of the yeast strains utilized in our experiments. This medium supported growth of the prototroph but not that of the adenine-requiring mutant. Final platings were made on a medium con-

sisting of 1% each of peptone and yeast extract, 0.5% monopotassium phosphate, and 2% each of glucose (or other carbohydrate source) and agar at *p*H 5.7.

Amphotericin B and its solubilized form (Fungizone) were kindly provided by E. R. Squibb & Sons, and endomycin by The Upjohn Co.

RESULTS

Effect of increased concentrations of antibiotic. Previous investigations on the selective action of antifungal agents (4) revealed that amphotericin B and endomycin were most effective at concentrations of 6 and 20 μg/ml, respectively, with an exposure period of 22 to 24 hr. However, large numbers of respiration-deficient variants survived this treatment, limiting the amounts which could be plated to 0.1 or 0.2 ml of the undiluted cell suspension. By employing membrane filters, samples of any size could be examined. Under these conditions, it was possible to use considerably higher concentrations of the selective agent, since the small number of survivors could be detected on membrane filters. By use of known mixtures of the adenine-requiring auxotroph and its parent prototroph, increased concentrations of amphotericin B and endomycin were tested for their ability to eliminate prototrophs and respiration-deficient mutants while permitting survival of the auxotrophs. Auxotrophic mutants were found to survive concentrations of endomycin ranging from 50 to 90 μg/ml, and concentrations of 90 to 100 μg/ml of amphotericin B. Significant increases in the final ratio of auxotrophs to prototrophs could be obtained by this procedure (Table 1). The tetrazolium-agar overlay technique revealed that few, if any, respiration-deficient mutants appeared under these conditions.

TABLE 1. *Effect of high concentrations of amphotericin B and endomycin on the survival of auxotrophs and protrophs*

Antibiotic	Concn	Initial auxotroph-prototroph ratio[a]	Final auxotroph-prototroph ratio[a]	Increase in auxotroph-prototroph ratio[a]
	μ/gml			
Endomycin	50	1:1	6:1	6 ×
	80	1:1	5:1	5 ×
	90	1:1	11:1	11 ×
	90[b]	1:1	83:1	83 ×
Amphotericin B	90	1:217	1:11	20 ×
	100	1:217	1:9	24 ×
	100[b]	1:1000	1:88	11 ×

[a] Expressed to nearest integer. Final plating on membrane filters.

[b] Period of aeration prior to addition of antibiotic and nitrogen source extended from 6 to 24 hr.

Effect of carbohydrate source. Respiration-deficient mutants have been reported to be unable to grow when certain carbohydrate sources are employed in place of glucose (3). Since preliminary experiments indicated that respiration-deficient mutants did not appear in significant numbers when yeast cultures were plated on agar containing galactose as the carbon source, experiments were conducted in which the selective action of endomycin was tested with various combinations of glucose and galactose in the medium for the inoculum culture, in the selection medium with endomycin as the selective agent, and in the final plating medium. None of the combinations tested appeared to be superior to glucose (Table 2). Lactate, acetate, and ethyl alcohol were found to be unfavorable for the normal growth and development of the pink pigmentation of the

TABLE 2. *Comparison of glucose and galactose as carbon sources for the selection of auxotrophs with endomycin (50 μg/ml) as the selective agent*

Carbohydrate[a] in			Initial auxotroph-prototroph ratio[b]	No. respiration-deficient (×10⁴)	Final auxotroph-prototroph ratio[b]	No. respiration deficient (×10⁴)	Increase in auxotroph-prototroph ratio[b]
Inoculation medium	Selection medium	Plating medium					
Glu	Glu	Glu	1:110	1	4:1	9	440 ×
Glu	Glu	Gal	1:39	1	6:1	3	234 ×
Glu	Gal	Gal	1:26	6	1:4	76	7 ×
Gal	Gal	Gal	1:10	3	1:2	81	5 ×
Gal	Gal	Glu	1:42	0	1:2	9	21 ×
Gal	Glu	Glu	1:18	0	1:3	25	6 ×
Gal	Glu	Gal	1:29	0	1:3	27	10 ×
Glu	Glu	Glu	1:33	8	1:3	77	11 ×

[a] Glu, glucose; gal, galactose.

[b] Expressed to nearest integer.

TABLE 3. *Effect of acriflavine (2.6×10^{-6} μg/ml) on conversion of prototrophs to respiratory deficiency*

Test	No. of auxotrophs ($\times 10^4$)	No. of prototrophs ($\times 10^4$)	No. respiration-deficient ($\times 10^4$)	Auxotroph-prototroph ratio[a]	Increase in auxotroph-prototroph ratio[a]
Initial	6	58	4	1:10	
	5	33	1	1:7	
Final	5	32	132	1:6	1 ×
	2	4	103	1:2	3 ×
Prototroph control					
Initial	—	128	7		
Final	—	2	140		
Auxotroph control					
Initial	57	—	0		
Final	44	—	76		

[a] Expressed to nearest integer.

adenine-requiring auxotroph; consequently, further evaluation of these carbon sources was not attempted.

Effect of acriflavine on surviving prototrophs. Ephrussi et al. (1) demonstrated that rapidly multiplying yeast are readily converted to respiration-deficient cells by treatment with acriflavine. Under the assumption that nonmultiplying auxotrophs would not be materially affected by acriflavine, attempts were made to render all surviving prototrophs respiration-deficient so that only respiration-sufficient cells would be considered as potential auxotrophic mutants. In practice, it was observed that the concentration of acriflavine required to achieve maximal conversion of prototrophs to respiratory deficiency also caused auxotrophs to give rise to respiration-deficient cells, even though they were unable to undergo multiplication under the conditions employed (Table 3).

Conditions favoring the action of antifungal agents on prototrophs. A mixed population of auxotrophs and prototrophs was inoculated into a minimal medium devoid of a source of nitrogen, and was aerated for a short period of time (ca. 30 min). Addition of ammonium sulfate did not immediately affect the viable count of either wild types or auxotrophs.A lag period of from 2 to 3 hr occurred before the wild-type cells entered the exponential growth phase (Fig. 1). Auxotrophs were found to survive at approximately the same level as in the initial inoculum for a period as extended as 24 hr. The results of several experiments revealed that addition of an antifugal agent after commencement of active proliferation resulted in maximal reduction of surviving wild types. As shown in Fig. 1, the addition of endomycin after 3 hr of aeration following addition of the nitrogen source resulted in a significant

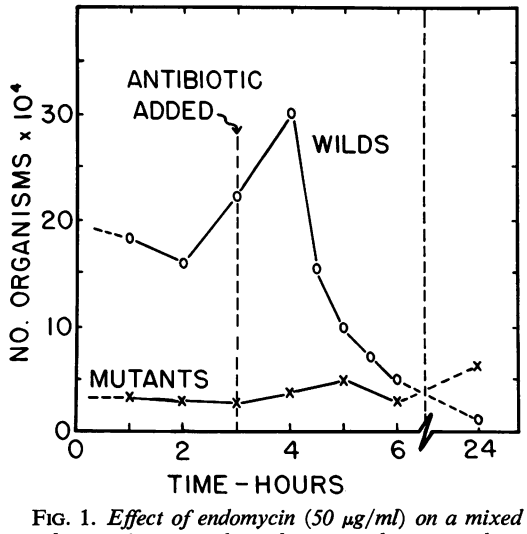

FIG. 1. *Effect of endomycin (50 μg/ml) on a mixed population of auxotrophs and prototrophs in a medium lacking the specific growth factor (adenine) for the auxotroph.*

reduction in the number of surviving wild types over the next 6 or 7 hr. However, maximal improvement in the ratio of surviving mutants to wild types was observed only after 24 hr of incubation. Addition of endomycin at the same time as the nitrogen source invariably reduced the selective action. Under these conditions, 50 to 80% of the colonies originally identified as wild types proved to be respiration-deficient by the tetrazolium-agar overlay technique.

Similar studies with amphotericin B yielded virtually identical results. However, preliminary studies with Fungizone revealed that neither auxotrophs nor protrotrophs survived overnight exposure. Fungizone very rapidly reduced the

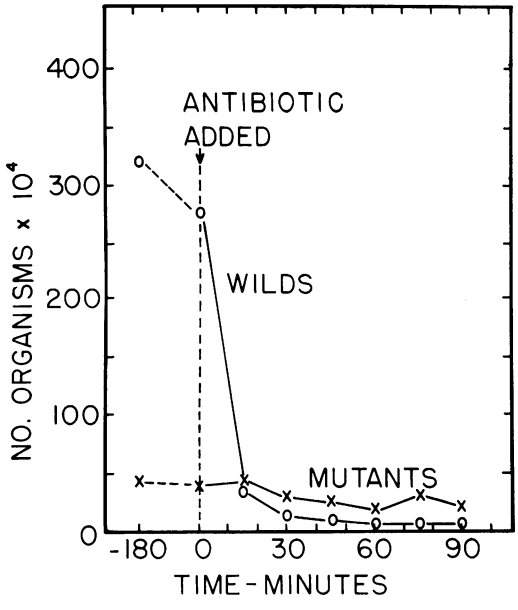

FIG. 2. *Effect of Fungizone (15 μg/ml) on a mixed population of auxotrophs and prototrophs in a medium lacking the specific growth factor (adenine) for the auxotroph.*

TABLE 4. *Effect of amphotericin B (Fungizone; 15 μg/ml) on survival of wild and auxotrophic yeast in mixed populations*

Time after antibiotic addition	Auxotroph-prototroph ratio[a]	Increase in auxotroph-prototroph ratio[a]	Auxotroph-prototroph ratio[a]	Increase in auxotroph-prototroph ratio[a]
min				
0	1:7	—	1:77	—
15	1:1	7 ×	1:4	19 ×
30	2:1	14 ×	1:2	39 ×
60	4:1	28 ×	1:5	15 ×

[a] Expressed to nearest integer.

wild-type population within the first hour of exposure (Fig. 2). The maximal selective action with Fungizone was achieved within 1 hr (Table 4). Furthermore, the selective action was equally pronounced at much lower initial mutant to wild-type cell ratios, and none of the surviving prototrophs was respiration-deficient. Undoubtedly, the short time required for elimination of wild-type cells under these conditions is unfavorable for the development of respiration-deficient variants.

DISCUSSION

Previous attempts to develop a selective procedure (4) for the isolation of yeast auxotrophs were hampered by the emergence of respiration-deficient variants in large numbers. By employing concentrations of endomycin or amphotericin B considerably in excess of those used in the earlier study, it was possible to isolate the small number of survivors on membrane filters. The final ratios of mutant to wild-type cells were quite satisfactory, and respiration-deficient mutants appeared to be unable to survive the higher concentrations of the antibiotics employed. However, the necessity of making multiple platings on membrane filters proved somewhat cumbersome and, in the absence of adequate sterile-room facilities, frequently resulted in excessive contamination.

The use of carbohydrate sources other than glucose did not provide a selective advantage of the auxotrophs over the respiration-deficient mutants. Ethyl alcohol, lactate, and acetate failed to support the growth of auxotrophs, and hence could not be employed. The substitution of galactose, which has been reported not to support the growth of respiration-deficient cells (3), was shown in our system to provide no selective advantage, since respiration-deficient mutants appeared when galactose was utilized as the sole carbon source. Acriflavine, in concentrations sufficient to convert all wild-type cells to respiration-deficient cells, converted a high percentage of the nonmultiplying auxotrophs to the respiration-deficient state. Thus, a procedure based on conversion of surviving wild-type cells to respiratory deficiency and subsequent screening with the tetrazolium-agar overlay technique could not be developed.

The rapid killing action of Fungizone and the delineation of the exact conditions under which maximal selective action could be achieved with this agent afforded the most effective procedure for the selection and subsequent isolation of yeast auxotrophs. No respiration-deficient mutants were found to be present under these conditions. This may be a manifestation of the increased efficacy of Fungizone against *S. cerevisiae*, which was similar to the effect obtained when high concentrations of endomycin or nonsolubilized amphotericin B were used. Alternatively, since the killing action of Fungizone occurs in less than 2 hr, as opposed to 22 to 24 hr with endomycin or amphotericin B, there may well be an insufficient number of divisions for respiration-deficient variants to emerge.

The conditions providing the best selective advantage include: (i) a short period of aeration in the basal medium in the absence of the nitrogen source or the antibiotic; (ii) addition of the nitrogen source and aerating until the wild-type cells begin to multiply rapidly (from 2 to 3 hr under our conditions); and (iii) addition of the antibiotic, followed by aeration for 1 to 2 hr.

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