SELECTION AND ISOLATION OF AUXOTROPHIC YEAST MUTANTS WITH THE AID OF ANTIBIOTICS[1]

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Yeasts have many advantages for studies in physiological genetics (see reviews by Beadle, 1945; Lindegren and Lindegren, 1949; Pomper, 1951). However, an efficient procedure for the elimination of standard type cells to facilitate the isolation of nutritional mutants has been lacking. The layer plate technique of Lederberg and Tatum (1946) has been employed successfully with yeast by Reaume and Tatum (1949) and Pomper (1949), but does not provide for the elimination of parent cells. Unfortunately, the penicillin selection method (Davis, 1948; Lederberg and Zinder, 1948; Adelberg and Myers, 1953), which has been so successful for the isolation of bacterial auxotrophs, has not been applicable to yeasts because they are refractory to penicillin. With the emergence of a number of antibiotics having high efficacy against yeast and other fungi, the possibility of applying one or more of these agents to the selection of vegetative yeast mutants was explored (Moat and Srb, 1957). Success in these preliminary investigations prompted us to continue to examine, with the help of known mixtures of parent and mutant cells, the selective potentialities of several antifungal agents for eventual use in the isolation of yeast auxotrophs.

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MATERIALS AND METHODS

Saccharomyces cerevisiae strain 174/1d, a wild, haploid yeast, and strain 173/1a, an adenine-requiring, haploid mutant, have been described by Ephrussi *et al.* (1949). Stock cultures of the two strains were maintained on a medium containing 1 per cent each of peptone and yeast extract, 0.5 per cent monopotassium phosphate, and 2 per cent glucose at pH 5.7. Two per cent agar was added for solid medium. The medium of Snell *et al.* (1940) was used for experiments requiring a basal medium of known composition.

The adenine-requiring mutant has been shown (Tavlitski, 1951) to develop a pink pigment when cultivated on agar medium in which the glucose concentration is increased. This color characteristic has been employed for visual identification of the adenine-requiring strain when plated in the presence of the wild-type culture. The ease with which the adenine-requiring strain could be recognized eliminated the necessity of subculturing each colony to determine its nutritional status, enabling evaluation of the nutritional status of all of the surviving colonies, and permitting a more critical assessment of the effectiveness of various agents and selection procedures in eliminating cells of the wild strain.

The antibiotics employed were provided by the following: Mycostatin (Nystatin), Amphotericin A, and Amphotericin B, from Drs. G. Peterson and E. Reed of Squibb and Company; Acti-dione, Filipin, and Endomycin from Dr. J. S. Evans of the Upjohn Company; Fungichromin from Dr. C. P. Hegarty of the Merck Institute for Therapeutic Research; Candicidin from the S. B. Pennick Company; and hexyl and heptyl esters of parahydroxybenzoic acid from Dr. M. Huppert, V. A. Hospital, San Fernando, California.

Stock solutions of 1.0 mg of antibiotic per ml of 95 per cent alcohol were diluted to 100 μg per

ml aqueous solutions with distilled water. All of the antibiotics except Mycostatin were sterilized by autoclaving for 10 min at 10 lb per sq in pressure at 115.5 C. Mycostatin was rendered ineffective by autoclaving and was therefore sterilized by filtration using Selas porcelain filters.

EXPERIMENTAL RESULTS

Development of the selection procedure. After a number of preliminary trials, the following general procedure was adopted. Ten ml of double strength basal medium, from which the ammonium sulfate was omitted, were added to each tube. For the inoculum, washed suspensions of the two strains were mixed in the required ratio, the mixture was diluted 1:10, and 0.2 ml was used to inoculate each tube. After a suitable period of aeration, the ammonium sulfate and antibiotic were added and the volume brought to 20 ml with sterile distilled water.

Since preliminary trials had indicated that Amphotericin B and Endomycin were most effective in eliminating cells of the parent type, these antibiotics were employed as the agents of choice in developing and improving the procedure. To determine the most suitable time for the addition of the antibiotic and the most effective incubation period in the presence of the agent, hourly plate counts were made on mutant-wild type mixtures following addition of the antibiotic. In one series, the antibiotic was added immediately. In the other, a 6-hr preaeration period preceded the addition of the nitrogen source and the antibiotic. Table 1 shows that preaeration in the absence of the nitrogen source greatly enhances elimination of the wild type cells while allowing survival of the adenine-requiring auxotroph. It is also evident that 11 to 12 hr exposure to the antibiotic is required before the selective action of Endomycin becomes evident.

TABLE 1

Evaluation of time of incubation and time of addition of the nitrogen source and antibiotic

Time of Incubation After Addition of Ammonium Sulfate and Antibiotic	Endomycin (20 μg/ml) and Ammonium Sulfate Added at Beginning			Endomycin (20 μg/ml) and Ammonium Sulfate Added After 6 hr Preaeration		
	Plate counts*		Nearest integer ratio M:W	Plate counts*		Nearest integer ratio M:W
	Mutants (M)	Wild types (W)		Mutants (M)	Wild types (W)	
hr						
0	10	22	1:2	19	40	1:2
2	13	53	1:4	19	43	1:4
10	25	22	1:1	43	30	1:1
11	24	13	2:1	48	17	3:1
12	12	8	2:1	58	5	12:1
22	3	10	1:3	34	1	34:1

* Average of the counts on four plates at each time interval. Values for the time intervals not shown were comparable to those shown and hence were omitted.

Amphotericin B was equally effective in eliminating prototrophs from the population while allowing survival of the adenine-requiring mutants. A 30-fold increase in the ratio of mutants to wild types was observed after overnight incubation.

Experiments in which the length of the preaeration period was varied from 1 to 12 hr revealed that beyond 4 to 6 hr, no marked improvement was afforded the selective action of either Endomycin or Amphotericin B. A preaeration period of 6 hr in the absence of the nitrogen source, followed by 22 hr exposure to the antibiotic in the presence of the nitrogen source, was therefore adopted as the standard procedure for subsequent experiments.

Selectivity of the agents with reduced numbers of mutant cells. In selecting mutants from a mixed population under the usual circumstances the number of mutant cells is small in comparison to the number of parent cells. Experiments were conducted in which the ratio of adenine-requiring mutants to prototrophs was greatly reduced. Table 2 shows the ability of Endomycin to eliminate the parent cells while allowing survival of the adenine-requiring yeast as the number of mutants with respect to the wild type was varied from 1:1 to 1:10,000. An 18- to 50-fold improvement in the M:W (mutant to wild type) ratio has been achieved over the range of ratios from 1:1 to less than 1:1000. However, the selective action appeared to be limited at ratios of 1:10,000 or less. A similar limitation was observed in the selective action of Amphotericin B.

Respiratory deficiency as the cause of limitation. Throughout the course of our studies it was repeatedly observed that many of the white colonies surviving exposure to the antibiotic agents were

TABLE 2

Selectivity of Endomycin with reduced numbers of mutant cells

M:W* Ratio (v/v)	Initial Count†		M:W Ratio (Actual)	Final Count†		M:W Ratio (Actual)	Increase in M:W Ratio	RD Colonies	M:W-RD Ratio	Increase in M:W-RD Ratio
	Ad⁻ M pink	Wild white		Ad⁻ M pink	Wild white					
1:1	134	189	1:1.4	1063	30	35.4:1	50×	29	1063:1	1063×
1:10	12	453	1:37.8	569	639	1:1.4	27×	522	5:1	190×
1:100	1	566	1:566	25	778	1:31	18×	633	1:6	94×
1:T	0	682	1:T‡	4	832	1:208	48×	399	1:108	93×
1:10T	0	501	1:10T‡	0	822	∞	None	745	∞	None

Endomycin, 20 μg per ml, and ammonium sulfate were added after 6 hr preaeration in the absence of antibiotic or nitrogen source. Final counts were made after 22 hr incubation at 30 C.

* M = mutant; W = wild type; Ad⁻ = adenine-requiring; RD = respiratory deficient; T = 10^3.

† Total count on four plates.

‡ Using the v/v ratio. The actual ratio would probably be less on the basis of the findings at the lower dilutions.

TABLE 3

Source of respiratory deficient colonies as indicated by independent exposure of the adenine-requiring mutant and the prototroph to Amphotericin or Endomycin

Time	Amphotericin B (6 μg/ml)					Endomycin (20 μg/ml)				
	Adenine-requiring mutant		Prototroph			Adenine-requiring mutant		Prototroph		
	Pink	White	Normal (N)	RD	N:RD	Pink	White	Normal (N)	RD	N:RD
hr										
0	120	0	178	8*	22:1	156	0	227	13*	18:1
22	1000	0	67	122*	1:2	1000	0	20	66*†	1:3

* Respiratory deficiency (RD) was assigned originally on the basis of small colony size.

† Of the 66 colonies designated RD on the basis of small colony size, 59 were found to be respiratory deficient colonies on the basis of failure to reduce triphenyltetrazolium chloride.

smaller than the usual colony size for this strain. This brought to mind the "petite colonie" respiratory deficient mutants described by Ephrussi *et al.* (1949). Using the triphenyltetrazolium chloride overlay technique devised by Ogur *et al.* (1957) for the identification of respiratory deficient colonies, 60 to 95 per cent of the white colonies surviving antibiotic treatment were found to be respiratory deficient.

To determine whether the respiratory deficient colonies were arising from the adenine-requiring mutant or from the prototroph, the two strains were independently subjected to the selective action of the antibiotics. Repeated testing of the two strains revealed that the adenine-requiring mutant did occasionally give rise to respiratory deficient colonies but did not produce them in the large numbers observed with the wild strain (table 3). The origin of the respiratory deficient colonies was also ascertained by determination of the nutritional status of colonies arising after exposure of the mixed population to antibiotic action. Most of the respiratory deficient colonies appeared to arise from the wild strain as evidenced by the fact that they grew in the basal medium without adenine.

Selective action of other antifungal agents. Once optimal conditions for assessing the selective action of Endomycin and Amphotericin B had been determined, seven other agents were tested for the ability to eliminate prototrophs from a mixed population. Of all of the agents tested, only Nystatin exhibited a selective action of the order of that observed with Endomycin and

TABLE 4

Comparison of selective action of several antifungal agents

Antibiotic	Conc	Initial Count			Final Count			
		Ad⁻ M*	Wild	M:W	Ad⁻ M	Wild	M:W	Increase
	μg/ml							
Amphotericin B	6	118	260	1:2	838	189	4.4:1	9×
Nystatin	2	41	111	1:3	221	29	8:1	24×
	3	14	31	1:2	26	1	26:1	52×
Acti-dione	1	57	103	1:2	61	203	1:3	None
	7	74	132	1:2	437	706	1:2	None
	10	16	38	1:2	700	344	2:1	4×
Amphotericin A	6	114	237	1:2	22	150	1:9	None
	5	18	37	1:2	12	124	1:10	None
Fungichromin	2	55	114	1:2	21	317	1:2	None
	2	24	27	1:1	2	9	1:5	None
Filipin	4	97	182	1:2	0	26	∞	None
	3	19	35	1:2	48	976	1:20	None
Candicidin	1	25	22	1:1	42	147	1:4	None
	2	27	44	1:2	242	115	2:1	4×
n-Heptyl-*para*-hydroxybenzoate	10	4	11	1:3	3	5	1:1	3×

Conditions were the same as for previous tables.

* Ad⁻ = adenine-requiring; M = mutant; W = wild.

Amphotericin B. However, three of the agents (Acti-dione, Candicidin, and *n*-heptyl-*para*-hydroxy-benzoate) exhibited some selective action (table 4).

DISCUSSION

Employment of an adenine-requiring mutant of *S. cerevisiae*, readily distinguishable from prototrophs by its pink pigment, has made possible the development of a method for assessing the ability of a number of antifungal agents to eliminate wild-type cells from a mixed population of mutants and prototrophs. Amphotericin B and Endomycin were found to be especially effective as selective agents. A 6-hr preaeration period in the absence of the nitrogen source followed by overnight incubation in the presence of the nitrogen source and the antibiotic provided optimal conditions for their selective action. At ratios of less than 1 mutant per 1000 wild type cells, these antibiotics still afforded survival of mutants while eliminating wild types.

The limitation of the selective action at lower mutant to prototroph ratios was found to result from the production of large numbers of respiratory deficient mutants which survived exposure to the antibiotics. These variants apparently survive antibiotic exposure in much the same manner as the adenine-requiring auxotrophs, either because they do not multiply under aerated conditions (Marcovich, 1951) or because the antibiotics are active only against respiratory mechanisms. The selective survival of respiratory deficient mutants in such large numbers restricts the procedure as it places a practical limit upon the aliquot which may be employed in plating the survivors. Since the respiratory deficient colonies can be readily identified by the triphenyltetrazolium chloride overlay method, they can be eliminated from consideration (see last part of table 2) and then the selective action of Endomycin can be shown to be much greater than the total survivors would indicate. The selective procedure could be of practical value if used with strains exhibiting a low frequency for the production of respiratory deficient mutants and the triphenyltetrazolium chloride overlay technique applied. The information obtained in these studies suggests that the method would be most applicable to strains which do not give rise to respiratory deficient mutants. This possibility was not explored, since it was found that all the haploid yeast strains in our collection produced respiratory deficient mutants in fairly high frequency.

Nystatin was the only other antibiotic which afforded a selective action comparable to that of Endomycin or Amphotericin B. As compared to the other antibiotics, Nystatin may afford special advantages because of its somewhat greater solubility in water. Whereas its greater lability could prove disadvantageous in maintaining active preparations, its lability might facilitate elimination of the antibiotic residual following treatment. Acti-dione, Candicidin, and *n*-heptyl-*para*-hydroxybenzoate exhibited sufficient selective action to warrant further study, however.

SUMMARY

A procedure has been developed to assess the ability of antifungal agents to eliminate wild type cells from a mixed population of mutant (adenine-requiring) and wild type yeast. Amphotericin B, Endomycin, and Nystatin exhibited a high degree of selective action under the conditions employed. Respiratory deficient mutants survived exposure to the antibiotics, placing a limitation on the use of the selective procedure with strains which produce these mutants at high frequency.

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