A DIFFERENTIAL AND DIAGNOSTIC PLATING METHOD FOR POPULATION STUDIES OF RESPIRATION DEFICIENCY IN YEAST[1]

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Small colony size ("petite") on glucose limiting agar has been associated with respiration deficient yeast by Ephrussi, Hottinguer, and Chimenes (1949), and Slonimski (1949), and has proved extremely useful in their tentative recognition and isolation. It has been apparent, however, that colony size is a reflection principally of growth rate. It might be anticipated that slow growth and small colony size would not serve as a universally reliable index of respiration deficiency.

This anticipation is borne out in yeasts bearing a number of nutritional deficiencies or following ultraviolet irradiation. In these instances small colonies have been found in this laboratory, some of which are still respiration sufficient. (See Raut, 1954, for comparison). Marcovich (1953) also found small colony size inadequate in population studies of respiration deficiency induced by acridines.

Most laboratories have devised some additional criteria for the confirmation of the phenotype. In Ephrussi's laboratory the Nadi test or spectroscopic examination have been employed (Slonimski and Ephrussi, 1949). In our laboratory 2 test procedures have been based upon the inability of respiration deficient (*aer*) yeasts to utilize certain organic acids as principle carbon and energy sources for growth. The first of these is based upon an acetate utilization-indicator color change test (PAC test) and the second upon a lactate nutrient agar which enables respiration sufficient (AER) cells to form colonies whereas *aer* cells do not (Ogur, Lindegren, and Lindegren, 1954). Raut (1954) has also observed that *aer* yeast failed to form colonies on a no-sugar nutrient agar whereas AER cells did and attributed the difference to the ability of the AER cells to use as a carbohydrate source "some carbohydrate derivative present in the yeast extract that is unavailable to the cytochrome-deficient cells." Raut has employed the no-sugar agar as a qualitative confirmatory test of the *aer* phenotype. She has, however, called attention to the observation that "in spite of the fact that deficient cells are unable to grow on no-sugar medium, when the same number of irradiated cells were streaked on nutrient agar plates with and without sugar, the same number appeared on both plates. This occurred despite the fact that half the colonies on the plate containing the sugar were cytochrome deficient."

Attempts have been made to employ tetrazolium salts in devising direct diagnostic plating methods for population studies of respiration deficiency. Laskowski (1954) attempted to perfect a diagnostic technique based on Raut's observation (1953) that a white colony on triphenyl tetrazolium chloride (TPTZ) nutrient agar proved to be *aer* whereas AER colonies were rose colored. Laskowski found, however, that certain concentrations of TPTZ induced *aer* colonies at high frequency, which of course, precluded its use for diagnostic purposes. Our own experience with a number of tetrazolium derivatives under a variety of conditions has thus far failed to yield a general diagnostic plating medium adequate to quantitative population studies.

The present report describes a quantitative method for the analysis of mixed populations of AER and *aer* cells based upon two plating media. The first, a control medium, allows both AER and *aer* cells to form colonies. The second, a selective medium, allows only AER cells to form colonies. In principle, therefore, a differential colony count of a mixed population of discrete cells spread upon two such plating media yields the proportion of AER and *aer* cells in the population.

EXPERIMENTAL METHODS

Yeast strains. The yeast strains utilized in the current work were from the collection main-

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tained in this laboratory. These are carried routinely as refrigerated oil-covered slants on a nutrient medium comparable to the control medium described below. Strain 11294 × 11296, a tetraploid hybrid which has been studied extensively, was chosen as a model AER yeast because of the relative ease with which it may be obtained as a population of discrete cells with few buds. Strain 11294-S-3 × 11296-S-1, a respiration deficient tetraploid obtained by mating spontaneous respiration deficient clones of the diploid parental strains 11294 and 11296, was chosen as a model *aer* yeast. It also showed no clusters but the budding percentage was generally appreciable under the growth conditions. Strain F-1, a clonal isolate from commercial bakers' yeast (Fleischmann's) served as a diploid model AER yeast.

Media. The *control* medium contained the following composition per L: Peptone (Difco), 3.5 g; yeast extract (Difco), 3.0 g; KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7 H_2O$, 1.0 g; $(NH_4)_2SO_4$, 1.0 g; glucose, 20 g; agar, 30 g.

The selective medium differed only by the substitution of sodium lactate (approximately 1 per cent end conc. pH 5.5) for the glucose.

Glucose or lactate liquid nutrient media were prepared as above except for the omission of the agar.

Growth of cultures. All cultures were taken as loop inocula from refrigerated oil storage slants. Fresh 24-hr slants were prepared and used as inocula for 40 ml of 2 per cent glucose liquid nutrient medium in 250-ml Erlenmeyer flasks. After 20–24 hr of shaker growth at 30 C, cells were harvested by refrigerated centrifugation, washed 2 times with distilled water, and diluted to a standard turbidity using the Klett-Summerson photocolorimeter. Serial dilutions were prepared with sterile water to a concentration 500 to 1000 cells per ml; 0.1 ml aliquots were spread by a bent glass rod on the surface of agar plates poured 24 hr before inoculation. Plates were covered with sterile unbleached muslin strips and allowed to incubate at room temperature (25–30 C) without being moved.

Glucose plates were scored at 2 to 3 days and lactate plates at 3 to 4 days. Rescoring plates at the end of 5 days did not reveal a significant increase in the number of visible colonies.

RESULTS

The qualitative observation that *aer* yeasts fail to form colonies on lactate agar was reported earlier based upon the examination of a few strains (Ogur, Lindegren, and Lindegren, 1954). To test the general qualitative applicability of the selective plating medium 50 AER strains and 50 *aer* strains were spread upon lactate nutrient agar and upon glucose nutrient agar. All *aer* strains failed to form colonies on the selective medium but did so on the control medium. Only one of 50 AER strains has thus far failed to form colonies on lactate nutrient agar. This aberrant strain is being investigated separately for a specific block in the pathway of lactate utilization. As a further test of the selective medium 710 yeast colonies were picked from selective plates on which mixtures of AER and *aer* cells had been spread. These colonies were transferred to glucose nutrient slants, which, after 24 hr, were used to inoculate PAC tubes. All yielded the color change characteristic of the AER phenotype, standardized against Warburg experience with glucose as substrate (Ogur, Lindegren, and Lindegren, 1954).

Insight into the biochemical rationale of the selective plating medium is provided by Warburg studies of AER and *aer* yeasts with glucose and lactate as substrates. Representative data are shown in table 1 for the model *aer* and AER yeasts referred to above. The *aer* yeast ferments glucose equally well in air or under nitrogen but does not oxidize it appreciably; it fails to show significant gas exchange with lactate as substrate either in air or under nitrogen. The AER yeast oxidizes glucose, ferments it both aerobically and anaerobically, oxidizes lactate with an R. Q. close to 1, but shows no significant anaerobic gas exchange with lactate.

The quantitative applicability of the selective medium has been tested in a group of model experiments. Aliquots of suspensions of the AER prototype were spread upon the surface of the control and selective media. In 3 experiments involving the scoring of 14,795 colonies on 240 plates the per cent AER estimated for the tetraploid AER model yeast (colonies on the selective medium/colonies on the control medium) was 99.6 per cent (±2.6 per cent s.d.). In 3 experiments involving the scoring of 9,240 colonies on 100 plates the per cent AER estimated for the diploid AER model yeast was 101 per cent (±4.4

TABLE 1

Comparison of respiration and fermentation of aer and AER yeast on glucose and lactate substrates

Yeast		Substrate					
		Glucose			Lactate		
Type	Strain	$Q_{O_2}(N)$	$Q_{CO_2}(N)$	$Q_{CO_2}^{N_2}(N)$	$Q_{O_2}(N)$	$Q_{CO_2}(N)$	$Q_{CO_2}^{N_2}(N)$
aer	11294-S-3 × 11296-S-1	ca. 0	3159	3168	ca. 0	ca. 0	ca. 0
AER	11294 × 11296	1259	2260	2033	491	451	ca. 0

Conventional Warburg procedure was employed. Substrate concentration was 0.5 per cent, pH 4.6, temperature 30 C.

TABLE 2

Model experiment III with AER and aer yeasts plated on control and selective media

	aer Colonies		AER Colonies		*aer* + AER Colonies	
	Total	Per plate	Total	Per plate	Total	Per plate
Control medium........	1783	44.6 ± 6.8*	1748	43.7 ± 8.5	3538	85.7 ± 11.4
Selective medium.......	0	0	1772	44.3 ± 7.5	1799†	45 ± 9.9

aer = 11294-S-3 × 11296-S-1, AER = 11294 × 11296, based upon 240 plates, 40 in each series.

* s.d. = $\sqrt{\frac{\Sigma d^2}{N - 1}}$.

† All colonies tested were AER.

per cent s.d.). The *aer* prototype grew well on the control medium but failed to show any visible colonies even after 11 days' incubation on the selective medium inoculated with 10^2, 10^3, 10^4, 10^5, or 10^6 *aer* cells per plate respectively.

Mixtures of AER and *aer* cells were prepared and tested on control and selective media. Data for a representative run are summarized in table 2. In this run 2 initial suspensions of *aer* and AER cells containing about 1000 cells per ml were used to prepare 3 suspensions by mixing aliquots of each with the other and with equal volumes of sterile water. Then 0.1-ml aliquots of each of the 3 suspensions were spread on both control and selective media, yielding 6 series of plates, 40 replicates to a series. Dilutions have so been arranged that *aer* plus AER mixture plates should represent the sums of the corresponding series of *aer* and AER cells plated alone. Variance between individual plates has been expressed as the standard deviation of an individual plate count from the arithmetic mean of that series. Tests for statistical significance reveal that it is extremely unlikely that the differences between mean plate counts of the

TABLE 3

Analysis of frank mixtures of AER and aer yeast by differential plate count

Experiment	Per cent AER	
	Calculated	Found
III	49.	51.
IV	8.2	8.7
V	21.	21.
VI	71.	67.
VII	88.	81.
VIII	87.	84.
IX	0.76	0.70
X	0.076	0.066
XI	0.081	0.090
XII	0.0081	0.0086
XIII	42.2	41.7

All plates were prepared by spreading 0.1 ml aliquots yielding ca. 50–100 colonies per plate. Percentages *calculated* are based on colony counts of 20–40 control plates of AER and *aer* suspensions before mixing, corrected for the dilutions. Percentages *found* are based in each series on colony counts of 20–40 control and 20–40 selective plates of prepared mixtures.

AER control, AER selective, and the *aer* plus AER selective series are significant, whereas the difference between the *aer* plus AER control and selective series is highly significant.

Results covering a wide range of quantitative compositions of *aer* plus AER mixtures are summarized in table 3. These results are expressed as the *per cent* AER *calculated* from control plates of AER and *aer* suspensions before mixing (corrected for the dilutions), and the *per cent* AER *found* as the ratio of the colony counts of selective and control plates. Different dilutions of the various mixtures were plated in each case so that the number of colonies per plate in most cases fell between 50 and 100. Good correspondence was found over the range studied. Even a small proportion of AER cells in a predominantly *aer* population (less than 1 in 10,000) could be estimated. More extreme ratios were not tested but there is no reason to believe that this represents the lower limit of the selective medium. *A priori* considerations would suggest that low concentrations of *aer* cells in a predominantly AER population could not be estimated with high precision by the proposed method.

DISCUSSION

Media based upon a principal carbon and energy source which is obligately aerobic for growth of Saccharomyces are selective for AER cells in mixed populations of *aer* and AER phenotypes. Experiments with 50 *aer* strains and 50 AER strains indicated that whereas all grew on a glucose nutrient agar, none of the former and all but 1 of the latter grew on a lactate nutrient agar. Where mixtures of *aer* and AER strains were spread on the selective medium, only AER colonies were found in more than 700 tested.

A number of qualitative features of such a selective medium are worth stressing. Most confirmatory tests of the AER phenotype are rapidly diluted out in mixed populations. It is questionable whether less than 10 per cent of AER cells in a mixed population would be reliably detected by measuring oxygen uptake in the Warburg respirometer, and the Nadi and spectroscopic tests might be expected to yield ambiguous results at even higher levels. The use of a selective plating medium makes it possible to detect, recover and estimate less than 1 AER cell in 10,000 *aer* cells. More extreme ratios have not been tested. The combination of selective and non-selective test procedures may be expected to yield necessary insight to the study of mixed populations arising from changes of phenotype either spontaneous or induced.

Quantitative applicability of the selective medium to the estimation of the proportion of AER cells in a mixed population based upon differential colony counts of selective and control plates has been demonstrated over wide population ranges with a model system. A differential plate count method for population studies may be expected to afford a major factor in convenience compared to individual clonal analysis in examining an adequate statistical sample of the enormous cell populations in microbial cultures. The limits of its applicability are worth noting however. A differential method may be expected to be susceptible to most precise application where the difference measured is large. It is generally most appropriate to contain this difference in the dilutions so that the number of colonies on control and selective media is approximately the same. Differential methods may be expected to yield greater errors where the difference measured is small. A population frequency of 1–2 per cent of *aer* cells (encountered spontaneously in some AER populations) is considered below the range of precise applicability of a differential plate count method, requiring instead a single, diagnostic plating medium for direct clonal analysis.

Attempts to apply tetrazolium compounds have been made. The observations of Laskowski (1954) concerning the induction of *aer* colonies by TPTZ have been qualitatively confirmed with some of the Carbondale strains which yielded sectored colonies on TPTZ agar. Dark-colored sectors were still AER, whereas light sectors were *aer*. Quantitative differences between Laskowski's observations and our own with respect to TPTZ toxicity and inducing concentration may be due to differences in strains or TPTZ sample. Neotetrazolium (NT) chloride did not induce the *aer* phenotype in our strains and was therefore seemingly better adapted to application as a direct, diagnostic plating procedure. Studies were made of the effects of dye concentration, pH, concentration of carbon and energy sources both facultative and obligately aerobic,

and other variables affecting the color differential between AER and *aer* colonies. Although several media recipes proved well adapted to qualitative distinction of *aer* and AER clones as heavy streak procedures, none has thus far proved adequate to the needs of a general direct and diagnostic plating method for quantitative studies of mixed populations.

The budding habit of yeasts creates some problems for all plating methods of population analysis. Particularly with haploids, the tendency of buds to remain attached leads to clustered growth and colonies based upon the cluster rather than the cell as a plating unit. Plating experiences with a series of AER haploids on the control and selective media indicated that the vigor of spreading and the consistency of the agar were factors leading to considerable variability in the number of colonies per plate. Although minimal spreading gave reasonable agreement between control and selective plates for AER haploids, there is little doubt that plating data with badly clumped haploids could be difficult to interpret at the cellular population level since only a single AER cell in a large cluster of *aer* cells would yield a colony on both control and selective media. It is, however, possible to select haploids showing little or no clustering or to employ hybrid strains at diploid, triploid, or tetraploid levels in essentially unbudded and unclustered condition for population studies of various agents inducing respiration deficiency with high frequency.

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SUMMARY

A differential plating method has been devised for studies of mixed populations of respiration sufficient (AER) and deficient (*aer*) yeast. The method employs a control medium enabling both AER and *aer* cells to form colonies, and a selective medium enabling only AER cells to form colonies. Model experiments with mixtures of AER and *aer* yeasts in various proportions indicated good correspondence between calculated and found population percentage over a wide range of compositions, even at low concentrations of AER cells in a predominantly *aer* population (where the differential count is large). The precision of the differential method becomes poorer as one approaches the other population extreme of low concentration of *aer* cells in a predominantly AER population (where the differential count is small). The selective medium may be applied qualitatively to the identification and isolation of a few AER cells in a predominantly *aer* population.

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