

A rapid, easy and economical method for assay of drug resistance in *Neurospora*

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Assay of bacterial drug resistance with linear concentration diffusion gradient plates was first described by Szybalski (1952 Science 116:46-51). We have applied the method to *Neurospora*.

Quantitative drug resistance may be estimated by measurement of the time-dependent increments of biomass in liquid cultures or extensional growth on race tubes with a series of concentrations at several culture time periods; however, we observe that wild type can acquire physiological resistance to many diverse drugs; therefore, it is necessary to make several measurements over periods of days to more than a week. Under those conditions, the potential maximal resistance (PMR), the least concentration that completely inhibits growth, is uncertain because the accuracy of the measurements decreases as growth increments approach zero. The relative degrees of wild-type and mutant resistance may be estimated by a judicious or arbitrary choice of one culture time period and interpolation of the data to the concentration that gives 50% inhibition.

Unlike the foregoing classical methods, the present one ideally requires only one small culture vessel per strain and relatively few measurements for a few days. Moreover the method not only permits (ideally) an accurate of PMR, but also may be useful for the selection of drug resistant mutants or revertants of drug sensitive mutants.

The method exploits the extensional growth habit and may be applicable to other filamentous fungi. Conidia are put on the "zero" concentration side of a gradient plate where they germinate to establish a mycelium that adapts to grow across the gradient. The concentration at which growth ceases is defined as the PMR. Thus, the procedure differs fundamentally from classical ones where conidia are immediately challenged with a bolus of drug. Under the latter condition, the drug may be uniquely lethal or toxic to conidial germination and/or resistance adaptation; hence the final results may not necessarily be related to the mycelial PMR.

Vogel's minimal medium with 2% each of sucrose and agar is sterilized by autoclaving and cooled to 55°C in a water bath before the addition of a drug. In preliminary experiments, several media, differing in drug concentrations by factors of 10, are prepared to determine the appropriate gradient concentration range for measurement of wild type's resistance.

Sterile plastic 5 cm diameter Petri dishes are tilted on a flat table in a sterile transfer hood by setting them on the edge of their lids. 5 ml of drug medium are put in a plate. The upper edge of the plate is marked with a felt pen to record the center of the lower end of the gradient. After the medium has solidified, the plate is set horizontally. 5 ml of drug free medium are added and allowed to solidify. The plates are inverted, set for a day to insure establishment of the diffusion gradient, inoculated with about 10⁵ conidia in 20 µl of water in a narrow streak along the lower side of the gradient at the dish's edge and incubated at 35°C in a humid chamber.

The distance from the edge of the plate to the mycelial frontier is measured on and about the diagonal across the center. With an appropriate gradient, strains with various degrees of

resistance cease to grow after two to six days. At that time, the distance to the front is a measure of PMR. The mycelial front in the central portion of the plate is usually quite uniform; hence estimate of PMR has an error variance of 10% or less.

Plots of growth distance at time intervals before PMRs are reached are curvilinear and resemble second-order decay kinetics. The transient degrees of resistance of various strains appear to be related to their PMRs in a regular fashion; however, we have not attempted to analyze the mathematical relationship.

By incorporating a dye in the medium (such as methylene blue) and photometric scans of the plates, exact measurements of the gradient linearity and its time-dependent change by lateral diffusion can be obtained (Szylbalski, pers. comm.). We have not conducted such measurements because we were only interested in the relative and not the absolute potential maximal resistances of wild type and mutants.

Age-1 and *age+* mutants are respectively inferior and superior to their wild-type parent in constitutive activities of at least 12 antioxyenzymes (Munkres et al. 1984 Mech. Age Dev. 24:83-100; Munkres 1990 Free Radical Bio. Med., in press). The accepted theory that those enzymes provide defense against free radicals and peroxides was confirmed. The PMRs of the strains with the oxidants paraquat, tert-butyl hydroperoxide and H₂O₂ were highly correlated with their enzyme activities (unpublished). (The theory of paraquat toxicity proposes that it generates toxic superoxide radicals in an enzyme catalyzed redox reaction.)

We conclude with technical observations related to the oxidants. On paraquat gradients, once growth ceases, usually no growth occurs for at least a week; however, on rare occasions hyphal strands grew ahead of the frontier and subsequently grew sideways. Those cells may be genetically-resistant mutants. In that context, it is probably significant paraquat has been reported to be a bacterial mutagen.

On a 0-50 mM H₂O₂ gradient, all strains quickly grew to the end; however on a 0-500 mM gradient, the PMRs were much less than 50 mM. Cells from the distal ends of the 0-50 mM gradients were transferred to 50-500 mM gradients whereupon the pMRs were 100-300 mM after 6-9 days. Those results apparently indicate that a 0-500 mM gradient is too steep to permit full expression of PMR without pre-adaptation on a 0-50 mM gradient. In contrast, the PMRs on 0-3 and 0-10 mM paraquat gradients were not significantly different.

On tert-butyl hydroperoxide, the PMRs of the strains were also significantly different; however, the ranges of PMRs differed on 0-1 and 0-10 mM gradients. In this case, the gradients are probably not linear. This peroxide is volatile at 35°C and its characteristic odor evolved from the plates for several days.

In conclusion, although there are minor problems in the interpretation of the assay results, such problems are probably also associated with classical assay methods. The rapidity, ease and economy of the method justify its use. - - - This research was supported by funds from the Laboratory of Molecular Biology. Contribution no. 3107 from the Department of Genetics.