How to enrich for mutations.

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Background

The original nutritional mutants of Beadle and Tatum were obtained nonselectively. A decade later, Woodward *et al.* (1954) and Catcheside (1954) adapted to Neurospora a selective method based on filtration enrichment. (The method was originally devised by Fries, 1947, using Ophiostoma.) Mutagenized conidia are incubated in liquid minimal medium, where prototrophs germinate and form hyphae. Vigorous shaking keeps cells in suspension and minimizes fusion and heterokaryon formation. The hyphal clots are removed periodically by filtration, while the ungerminated auxotrophic conidia pass through the filter. After several rounds of filtration, the remaining conidia are plated on complete medium under permissive conditions. Procedures for filtration enrichment were later refined, with substantial increases in efficiency (Case, 1963, Applegate *et al.* 1978, Yoder 1979). The most significant advance was introduction of sorbose into the incubation medium. The colonializing agent made hyphal elements more compact so that they do not trap ungerminated conidia, and it allowed successive filtrations to be made at intervals of 12 hours rather than 4. Temperature-sensitive mutants can be selected by imposing the appropriate temperature regime. In addition to auxotrophs, morphological mutants and chromosome rearrangements were found among the survivors.

Organic complete medium may inhibit some auxotrophs (e.g., histidine, homoserine, guanine) or may be inadequate for others (e.g., fatty-acid requirers that respond to Tweens). Temperature-sensitive threonine and asparagine mutants have mistakenly been scored as irreparable because they fail to grow normally on complete medium (Perkins and Ishikawa 1972). Thus, temperature-sensitive mutants not growing on complete medium at 34°C should be checked on minimal medium plus these supplements before concluding that they are irreparable.

Another effective method of enrichment, called 'inositolless death', was introduced by Lester and Gross (1959). In principle, this is analogous to the penicillin enrichment method used in bacteria. Its effectiveness depends on unbalanced metabolism, which leads to death of the inositol-requiring single mutant in absence of inositol. Acquisition of a second mutation slows metabolism and leads to survival of the newly arisen double mutant. Mutagenized conidia are incubated in medium without inositol until most of the cells are dead. The survivors are then plated in complete medium or medium with inositol and other supplements as desired, and the resulting colonies are tested on minimal plus inositol for new mutations. Although an inositol mutant is usually employed, other auxotrophs can be used. Those affecting membrane integrity are especially effective.

Special methods have been devised to select for particular categories of mutants. For example, nitrate nonutilizers can be selected by resistance to 1.5% potassium chlorate (see Marzluf 1997). The *mtr* and *am* genes allow both forward and reverse mutations to be selected. Mutations affecting different steps in the same biosynthetic pathway can be obtained efficiently as double mutants by filtration enrichment using a bradytroph (a strain already partially impaired in an enzyme of the pathway) (Davis 1979).

The question may be asked, whether these methods for obtaining mutations will any longer be used, now that the genome sequence makes reverse genetics possible. While enrichment methods will probably not be used for shotgun mutant hunts, as in the past, they should still prove useful for obtaining new alleles with specific properties (including conditionals), for obtaining mutant combinations that display novel phenotypes, or for other, unanticipated, objectives.

Procedure

Filtration enrichment. The following is taken from Yoder (1979).

" The high-sorbose, filtration concentration method of mutant enrichment reported by Applegate *et al.* (1978) was modified and evaluated for its efficiency of mutant selection. Conidia were suspended in water, filtered through four layers of cheesecloth (20 mesh/inch), adjusted to 2×10^6–2×10^7/ml, and 10 ml of the suspension was placed in a 10 cm diameter glass petri dish for a one min exposure to UV-light (48 ergs/sec/mm^2) that resulted in 70 to 90% kill. The suspension was transferred to a 500 ml flask containing 250 ml Vogel's minimal salts plus 6% sorbose, 0.5% glucose, and 0.5% fructose. The flask was incubated in a water bath shaker at 100 reciprocations/min; restriction of growth was greater at 37°C than at 25°C. At 12 hr intervals (at either temperature), the suspension was passed through a single layer of Nitex #53 nylon monofilament screen with 35 μm openings, (Baylis *et al.* 1965, Turtox 73-511-4); four layers of cheese-cloth also filtered effectively but were less convenient to handle. When the filtration was complete, nongerminated conidia were collected by one of two methods. 1) If the filtration medium contained no inhibitor, the suspension was mixed with an equal volume of warm (60°C) complete agar medium, or appropriately supplemented minimal, and poured into petri dishes (15 to 20 ml/dish). 2) To wash conidia, a dense suspension of "carrier conidia" (killed by 12 hr incubation at 60°C) was added, and the conidia were pelleted by centrifugation and resuspended in 15 to 20 ml water (additional washing by centrifugation was sometimes necessary). Carrier conidia aided in locating the pellet and reduced the likelihood that living conidia would stick to each other during centrifugation or would be lost in the supernatant. (Carrier conidia have previously been used in this way by D. E. A. Catcheside.) Aliquots (1 ml) of resuspended conidia were placed in petri dishes and mixed with warm agar medium.

After a 60 hr filtration period ot 37°C, there were 500 to 600 survivors, of which about 30% were auxotrophs; after 84 hr there were less than 100 survivors and up to 92 % auxotrophs. Averages from several experiments show that among all survivors, 11% were temperature sensitive, 57% were auxotrophic, and about 30% produced white ascospores indicative of chromosome aberrations, in tests carried out by D. D. Perkins. Specific selection for guanine auxotrophs or for caffeine sensitivity yielded 2% in each case.

Three isolates with Oak Ridge background, *al-2* (15300; FGSC 3448), *eas* (UCLAl91) and *eas;mts* (UCLA191; MN1), were compared for utility in mutant hunts. All three were equally easy to handle and were efficient for selection of auxotrophs; average mutant yields for two experiments with 84 hr filtration-enrichment periods were: *al-2* 89%. *eas* 73%, and *eas; mts* 92% (for each isolate a few of the auxotrophs were leaky and a few were morphologically abnormal).

The method is thus extremely efficient, in addition to being convenient. A modification of the method has been useful with another ascomycete, *Cochliobolus heterostrophus*. By replacing 1.5% glucose in the filtration medium with 2% sorbose and 0.2% glucose and adjusting procedures slightly, the rate of auxotroph selection was increased from about 0.1% to 1 to 2%."

Metzenberg (1989) suggests an improvement in the method of filtration:

"Suspensions containing conidia and growing hyphae may be separated by filtration through glass wool, cheesecloth, Miracloth, or absorbent cotton, but in my hands, the separation is erratic. The filter may either contain a few oversized holes so that everything passes through, or it may plug up so that even nongrowing conidia are removed. I find "Thermolam Plus", a felted synthetic, to be very much superior to the traditional filtration materials. It is distributed by Stacy Industries, Inc., P.O. Box 395, Wood Ridge NJ 07075-0395 (Cat. # 970), but it can be bought very inexpensively in small amounts at ordinary fabric stores. The Thermolam Plus is cut to the desired shape, put into the filter holder of choice, and autoclaved. The suspension to be separated is poured onto the filter. Non-growing conidia (or ascospores) in suspension pass through readily without clogging the filter, while those that have grown substantial hyphae are retained."

Enrichment by unbalanced growth.

Lester and Gross (1959) used the nonreverting *inositol* allele 89601. Sullivan and DeBusk (1971) simplified the procedure and used the method to select for new temperature-sensitive auxotrophs and mutants with defects that are irreparable by supplementation. To do this, they used the temperature-sensitive *inl* allele (83201t), which is killed by incubation at 34° or 35°C in absence of inositol, but which grows well on the same medium at 25°C. Survival of 83201t conidia at 34°C is about 1/10 after each 24 hours.

"A suspension of *inl* (83201t)*; ylo-1* conidia is mutagenized, plated on sorbose minimal medium, and incubated at 35°C for 36-40 hrs. The plates are then shifted to an incubation temperature of 25°. Colonies may be picked 2-5 days after the temperature shift. Isolates may then be tested for failure to grow on inositol-supplemented minima1 medium at 35°C. This method provides a strong selection for temperature-sensitive mutants, which will not begin to grow on minimal medium at 35°C (and therefore will not be killed in the absence of inositol) but which can grow on minimal medium at 25°C. The parental strain is killed by incubation at 35°C in the absence of inositol, and auxotrophic mutants that are not temperature-sensitive should not be able to grow on minimal medium at either temperature and therefore should not develop on the plates. Overlaying of plates with supplementary inositol-comtaining medium is unnecessary since *in*l *(83201*t*); ylo-1* does not require inositol at 25°C."

Strains for enrichment

al-3 inl (89601) FGSC 2308 *A*, 2301 *a*; and *al-3 inl* (83201t) FGSC 2309 *A*, 2310 *a* contain the *inl*-linked *albino-3* mutation, which serves to detect contamination and which may facilitate freeing new mutations of the inositol requirement.

Each of the two *inl* alleles has been combined with *sn cr-1* to provide the option of scoring by replication: *sn cr-1; al-3 inl* (89601) FGSC 2303 *A*, 2306 *a*; and *sn cr-1; al-3 inl* (83201t) FGSC 4160 *A*, 4161 *a*. (*sn cr-1* strains, which are female-fertile, form small conidiating colonies, making sorbose unnecessary).

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