How to activate ascospores and optimize the recovery of progeny.

Background

Wild-type ascospores remain dormant until activated by heat or chemicals. Dodge's serendipitous discovery that Ascobolus ascospores are activated by heat (Dodge 1912) served him in good stead when the sexual phase of Neurospora was discovered and heat activation was used to expedite genetic analysis, enabling him to perform the first tetrad analysis and to demonstrate Mendelian segregation of mating type (Shear and Dodge 1927). Not only did exposure to heat break ascospore dormancy, it also conferred the great advantage that conidia and mycelial fragments are killed, ensuring that all germlings growing up after heatshock originated from $f_1$ascospores rather than from surviving cells of partntal genotype.

Lindegren (1932, using *N. crassa*) and Goddard (1935, using *N. tetrasperma*) experimented with germination as a function of temperature and duration of treatment. Their results led to the adoption of 30 minutes at 60ºC as a standard treatment, giving optimal germination. Investigations of dormancy and germination were extended and reviewed by Emerson (1954) and by Sussman (1961, 1966, 1976; Lingappa and Sussman 1959)

Discovery of chemical activation by Mary Emerson (1948) followed the observation that germination occurred without heatshock on media containing xylose and that it was higher when the xylose medium was autoclaved than when filter-sterilized xylose was added after autoclaving. This led to identification of the breakdown product furfural as the effective agent. Later studies identified related compounds that are active (reviewed by Sussman 1966, 1976). Robert Metzenberg found that 2-furyl alcohol is less toxic than furfural, and he combined it with heat treatment in a protocol to activate ascospores and recover Neurospora and Gelasinospora from soil samples (Glass *et al*. 1990. See *How to sample natural populations*).

Procedure

Treatments used for activation

- *Heat shock*. Ascospores may be activated either before or after tubing or plating. In the Stanford lab they are usually isolated to 10×75 mm slants and heatshocked in a water bath. Alternatively, ascospores may be spread on an agar surface in plates, heatshocked, and isolated to slants following germination. Temperature control is better in a water bath than in a hot-air oven because of the greater specific heat of water and the lag in returning to temperature after an oven has been opened. All conidia and small mycelial fragments are killed in 30 minutes if a 60º water bath is used. Although an oven is more convenient for use with Petri plates and the lag in reaching 60º can be compensated for by longer exposure, more reliable temperature control is achieved with a water bath. Ascospores in plates can be heatshocked without difficulty in a covered water bath if a false floor is used that allows only the bottom of an uncovered plate to touch the water.surface.

    Lindegren's (1932) figure shows ascospore viability falling off gradually after 30 minutes at ~60ºC and plummeting only after 2 hours. If sizeable clots of mycelia are present,. water-bath exposures somewhat longer than 30 minutes would be advisable to ensure that all vegetative cells are killed.

    Warning: Neurospora ascospores and gemlings are highly sensitive to heat following activation by heat shock (Lingappa and Sussman 1959). Once spores are removed from 60ºC to a lower temperature, they should never be returned to the heat shock temperature.

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- *Chemical activation.*

  Unlike heatshock, chemical activation by furfural or related compounds does not kill conidia or other vegetative cells. One of the great advantages of using heat is thus forfeited. The problem of killing vegetative cells is usually solved by exposing ungerminated ascospores to a 1.% solution of sodium hypochlorite (Reagent grade) or a 20% dilution of commercial bleach such as Chlorox (sold as a 5% or 6% solution of sodium hypochlorite). Only then are the ascospores exposed to the activating chemical (Emerson 1948). Before being isolated, ascospores are spread over a 4% agar surface that has been soaked with the sodium hypochlorite.

  Warning: If the hypochlorite concentrationa is too high, the ascospores are bleached and killed..

  See Sussman and Emerson papers for details.

- *Combined heat and chemical* treatment.

  The following is from Glass *et al.* (1990):

  "Reconstruction experiments suggested that germination was improved by a combination of heat shock and a furane derivative as compared with either one alone, and that 2-furyl alcohol, unlike furfural, was not toxic to the cultures at concentrations moderately above the effective concentration. D-xylose at 1% gave substantially better germination than did sucrose, possibly because it gives rise to traces of furfural during autoclaving or during the heat shock; fructose was also effective, but slightly less so…. Germination of ascospores was excellent at pH 6.5 but very poor at pH 4.5."

Other factors affecting germination and recovery

- *Dehydration*. The following is based on Perkins and Strickland (1973):

  Ascospores from dessicated crosses germinate poorly if heat-shocked directly after harvest or isolation. (Extremely dehydrated spores may collapse and become crescent shaped.) The low germination can be overcome by rehydrating either in water or on the surface of fresh agar medium. When isolating from old cross tubes, we obtain good results by adding sterile water to the tube 12 hours before isolating.. Alternatively, perithecia or ascospores may be removed and suspended in water. Ascospores which had been stored dry for 27 days showed 31% germination before hydration. Germination was restored to 97.5% by rehydration (Strickland 1960).

  Rehydration is not necessary when our standard procedure is followed. Crosses are made on 10 ml slants in 19 × 150 mm tubes and ascospores are usually isolated before agar in the slant has shrunk away appreciably from the wall of the tube. Only if isolation is delayed until the agar has shrunk to half its original volume do we take steps to assure that ascospores are hydrated. With crosses in small tubes (10 × 75 mm or 12 × 100 mm), drying out occurs quickly and rehydration is more likely to be necessary.

- *Genotype*. Ascospore viability and germination rate are also affected by mutation of specific genes, or by deletions.

  Mutant genes resulting in spontaneous germination. The two best known examples are *fl* (*fluffy*) and *per-1* (*perithecial-1*). A significant fraction of ascospores containing the *fluffy* mutation germinate spontaneously. Once germinated, they are vulnerable to killing by heatshock, with the result that segregation ratios are distorted. Failure to recognize that distorted allele ratios are due to the death of spontaneous germinants could lead to misinterpretaion of results when *fl* is present in a cross. The

distortion can be minimized by using fresh crosses and by heatshocking immediately after ascospores have been placed on nutrient medium.

Ascospores containing Type 1 alleles of *per-1* remain unpigmented but are viable and appear not to be dormant. When placed on normal medium they germinate without heat shock and are killed by exposure to 60ºC. For methods of recovering *per-1* progeny that are uncontaminated by vegetative cells of parental genotype, see *How to obtain uncontaminated progeny from crosses involving per-1.*

Mutations that impair ascospore maturation or germination.

Genes with variable pleiotropic effects. Many mutants that were originally identified by an abnomral vegetative phenotype also affect ascospore maturation, either when homozygous (e.g., *ad-3A*, *ad-3B*, *arg-1*, *lys-3*) or in an ascospore-autonomous manner when heterozygous (e.g., *cys-3*, *le-1*, *le-2*, *lys-5* [= 'asco'], *pan-2*). Expression of some of these can be alleviated by adding supplement, others are not remediable. With some genes, defective ascospores ripen slowly and progeny can be obtained after ageing (e.g., ascospores containing the *sk* (*skin*) mutation give good allele ratios only after 3 weeks). At some loci (e.g., *lys-5*), mutant alleles of independent origin differ in their effect on ascospore development, with some alleles failing to blacken and mature while others are normal or nearly so. See Perkins *et al*. (2001) for these and other examples.

Rearrangement-generated segmental deficiencies. Ascospores carrying deficiencies of defined extent are generated in crosses that are heterozygous for insertional or terminal chromosome rearrangements. Usually the deficiency ascospores are unpigmented because they die before reaching maturity, and the deficient nuclei cannot be rescued in heterokaryons.. Barry (1992) has reported a short deficiency that does not prevent ascospores from maturing and germinating. The deficient germlings do not progress beyond the germination tube, but they can be rescued by adding a helper strain to form a heterokaryon (Perkins 1984. See *How to use helper strains for maintaining and crossing handicapped recessive mutants*.)

Recovering progeny of strains carrying recessive mutations that affect ascospore viability.

Special methods have been devised to recover progeny containing mutations that impair ascospore development and maturation. If the mutant ascospores mature enough to germinate, then recessive mutations can be rescued simply by adding a *het*-compatible *helper* strain, which fuses with the germling to form a heterokaryon. See *How to use helper strains for maintaining and crossing handicapped recessive mutants*. But ascospore development in many ascospore-lethal mutants is arrested at early stages, before germination, and *helper* strains cannot form a heterokaryon with an ungerminated ascospore. Using a different strategy, these early-arresting ascospore-lethal genes can be recovered if they are present in an ascospore that is already heterokaryotic before germination. Heterokaryotic ascospores can be obtained in either of two ways. A strain containing the deleterious recessive mutant can be crossed with a strain containing a dominant gene that results in meiotic nondisjuction (e.g., *Dip: Diploid ascospores*). Alternatively, it can be crossed with an ascus-development mutant that lays down ascospore walls enclosing two or more nonsister meiotic products (e.g., *Ban:Banana* or *Fsp: Four spore*). (See Perkins *et al*. 2001 or Radford's e-compendium http://www.bioinf.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm for a description of these mutants and for references.)

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