How to test for complementation between mutant strains.

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Background

Since the vegetative phase of Neurospora is haploid, functional tests of allelism cannot routinely be made by constructing heterozygous diploids but will usually depend on the ability of genes to complement one another in heterokaryons. Because Neurospora mycelia grow so rapidly and vigorously, positive complementation tests are usually clear and rapid.

Partial diploids could also be used for testing complementation, but constructing them is laborious and depends on the availability of suitable duplication-producing insertional or terminal chromosome rearrangements. (See Perkins 1997. See *How to use duplication-generating rearrangements in mapping*.)

Dodge (1942, Dodge *et al.* 1945) provided the first example of complementation in *N. tetrasperma*. Beadle and Coonradt (1944) explored complementation in heterokaryotic strains with multiple components, in hope of determining degrees of dominance. With the advent of efficient methods for selecting auxotrophic mutations, series of mutants with the same requirement were tested for allelism by complementation tests which used heterokaryons to place them in complementation groups that corresponded to different loci (*e.g.*, de Serres 1956, Webber and Case 1960). A similar analysis has used heterokaryon formation to place new morphological mutants in >100 different complementation groups (Seiler and Plamann 2003).

Ability to complement was used to distinguish multilocus deletions from gene mutations in large-scale studies of mutation in the *ad-3* region (*e.g.*, de Serres and Brockman 1999), to detect recessive lethal mutations (Stadler and Macleod 1984), and to use 'sheltered disruption' by RIP for determining whether a gene product is essential (Metzenberg and Grotelueschen 1992, Nargang *et al.* 1995).

Failure to complement provides a clear indication of allelism provided that strains being tested are heterokaryon compatible, as is expected for mutations induced in the same strain. Conversely, ability to complement usually indicates nonallelism, but exceptions occur. Occasionally, pairs of mutants that specify the same enzyme were found to be capable of complementing one another (Fincham and Pateman 1957, Woodward *et al.* 1958, Catcheside and Overton 1959). This was explained in terms of multimeric proteins. Many experiments were done in the 1960s using complementation between alleles ("allelic complementation") in the unsubstantiated hope of constructing meaningful complementation maps. Allelic complemention is pairwise between specific mutants. Occurrence of mutations that are incapable of complementing any other mutations in the same gene establishes that all of them are alleles. For history and a critique of the bearing of allelic complementation on Benzer's concept of the gene as a cistron, see Fincham (1966, 1977, 1988).

Procedure

For most purposes, especially where small numbers are involved, the simplest method for testing the ability to complement is to superimpose small quantities of mycelia and/or conidia at a spot on the surface of a large slant of minimal or other appropriate forcing medium. For large-scale testing, or when speed and efficiency of complementation are being investigated, special methods have been devised. Tests may use petri plates or tubes, solid medium or liquid, conidiaor mycelia. The following are examples.

"Heterokaryon tests were made with heavy conidial suspensions of the individual mutant strains. Approximately 0.03 ml of the individual conidial suspensions was placed in spots near the periphery of petri plates previously prepared with 15–20 ml of minimal medium solidified with 1.0% agar. Each plate contained control spots of the two strains being tested with each other, and two spots where conidia were mixed and heterokaryon formation could take place. The spots where the conidia were mixed were diametrically opposed so that when a positive heterokaryon test was obtained, the two heterokaryons grew toward each other, and did not rapidly overgrow the control spots. When heterokaryon tests were made on "leaky" strains, which grow to some extent on unsupplemented minimal medium, heterokaryon formation could be detected only by the difference in size of the colony formed on the control spot of the "leaky" strain versus the spots where conidia from both strains were mixed. All plates were incubated at 25°C and results recorded after 24–36 hours." (de Serres 1956)

"We used to use Petri plates containing a minimal sorbose medium on which 25 tests could be conducted simultaneously. This method has very largely been superseded by the use of tests done in 4" [100 mm] test tubes closed with Oxoid Caps. Both are described in Catcheside 1960. Proc. Roy. Soc. London B153:179; more detail of the plate tests is given in Ahmad and Catcheside 1960 Heredity 15:55. For the tests in tubes we use baskets holding 64 tubes in 8 rows of 8 each. Each basket is labeled in a standard fashion with a code number corresponding to the protocol of the matrix to be set up. Drops of conidial suspension are added to each by means of Pasteur pipettes. The medium contains agar, not sloped, so that the conidial mixture sits on the top and is easily visible on inspection. Daily records are kept for up to 10 to 14 days. Beyond this time the medium tends to dry out too much, so concentrating the constituents. The concentration of the medium as well as other factors such as the temperature of incubation, affects the ability to grow." (Catcheside 1964).

"Loops of conidial suspensions, or small spots of conidia carried on the tip of a moistened needle, were superimposed on the surface of plates containing minimal agar medium N with 0.4% sucrose and 0.9% sorbose to induce compact growth . . . Singly inoculated . . . mutants give little or no growth for 1 1/2–2 weeks or more; consistently positive growth was shown by complementary combinations of mutants within a week and within 3 days in the cases of the stronger combinations." (Fincham and Stadler 1965).

When studies of allelic complementation required large numbers of tests, special procedures were devised. The ultimate protocol (de Serres 1962) used racks of 100 75 × 100 mm tubes, which are not plugged. The racks are wrapped in a taut double layer of Saran Wrap, sterilized, and tubes are inoculated with 1 ml aliquots of conidial suspensions of the two strains being tested, using 2 ml Cornwall continuous pipetting outfits fitted with 21-gauge hypodermic needles. The Saran Wrap over each tube is pierced twice to introduce successively 1 ml of each

inoculum, inoculated vertically on opposite sides of the tube so as to avoid cross contamination. This efficient system made it possible to test many thousands of combinations (de Serres 1963).

Complementation tests of temperature-sensitive morphological mutants were carried out as follows (Seiler and Plamann 2003):

"Conidia of two strains were inoculated as a dense suspension separately and together on a plate, incubated overnight at 25°C to allow germination as well as fusion of the two coinoculated strains, and then shifted to restrictive temperature [39°C] to score for complementation. Heterokaryon incompatibility was not a problem because all mutants were derived from the same parental strain."

Validity of all negative tests depends, of course, on the strains being heterokaryon compatible. (See *How to identify and score genes that confer vegetative incompatibility*.) The quickest way to test for *het*-compatibility with Oak Ridge strains is to determine whether the strain in question can complement *helper-1* (Perkins 1984) or another of the available *N. crassa* helper strains. These all have forcing markers in OR background, and they are all *het*-compatible with both mating types. (See *How to use helper strains . . . for determining heterokaryon compatibility*.)

The growth rate of heterokaryons between strains with recessive auxotrophic forcing markers is wild type over a wide range of nuclear ratios, but decreases if one component is very rare. The following method for preparing heterokaryons with known nuclear ratios is adapted from Pittenger *et al*. (1955, 1956):

“Conidial suspensions from homokaryotic cultures 6 to 10 days old are mixed in various proportions. Mixtures are soaked overnight at 3°C in supplemented liquid medium to allow maximum hydration and absorption of growth factors without germination. The suspensions are then incubated 2 to 4 hours at 29°C in the same medium. The conidial mixtures are centrifuged to rather viscous pellets and the pellets (or several samples of each pellet) are incubated 3 days at 29° or 33°C on supplemented sorbose agar. The coalesced pellets are then transferred to minimal slants or growth tubes.”

When macroconidia are in close proximity, effective mixing of nuclei is assured by the formation of specialized 'conidial anastamosis tubes' that interconnect the conidia (Roca *et al*. 2004).

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