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Methods for the identification of genetic alterations by specific revertibility tests in Neurospora crassa

H. V. Malling

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Abstract
Revertibility tests

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The identification of the different types of genetic olterotions in a series of allelic mutants is particularly important for understanding gene-enzyme relationships. The approach that is being used to identify the genetic olterotions is to study the specific revertibility of individual mutants with the use of chemical mutagens whose mode of action is wi-pected or known. The use of nitrous acid (NA), ethyl methanesulfonate (EMS), hydroxylamine (HA) and ICR-170 (a monofunc-tional ocridine mustard) should permit one to distinguish the following three classes of genetic olterotions: (1) Single base-pair substitutions. Mutants which revert after treatment with either HA and/or NA and in many cases also after treatment with EMS. Some of the single base-pair sbustitions ore also revertible with ICR-170. (2) Single base-pair insertions and deletions. Mutants which revert only after treatment with ICR-170 and then usually with a very high frequency. In a few cores mutants in this cate-gory also give a slight increase over the spontaneous frequency after treatment with NA ond EMS. (3) Unidentified genetic olterotions. Mutants which revert only spontaneously or stable mutants which do not revert at all.

Preparation of the culture. In all experiments the mutagenic treatment was carried out on suspensions of conidio harvested from 125-ml Erlenmeyer flasks containing 20 ml of glycerol complete medium (10 ml glycerol per liter instead of 20 ml) + adenine sulfate (25 mg per liter). The flasks were inoculated and then incubated for 1 day at 30°C and then for 6-9 days at 25°C. The conidio were harvested by first shaking the cultures with gloss beads (4 mm diameter) to break up the chains of conidio; they were then suspended in saline (0.9 %), filtered through a platinum strainer, washed twice by centrifugation and then resuspended in saline. Purple mutants differ from each other with respect to the amount of the Purple pigment in the mycelium. However, when conidio ore grown on glycerol complete, supplemented with 250 mg adenine sulfate per liter, purple pigment accumulation is essentially eliminated. The density of the conidiol suspensions was measured on a colorimeter (Spectronic 20, Bausch and Lomb, Rochester, New York) at 750 mμ, where the absorption is maximal.

Treatments. All the treatments were carried out with conidiol suspensions (co. 2 x 10^7/ml) in Erlenmeyer flasks in a water bath at 25°C on a rotary shaker to keep the conidia in suspension during the treatment. Five minutes before quenching, the conidio were centrifuged and the supernatant was decanted. At the time of quenching, after treatment with either NA, EMS or ICR-70, the conidio were resuspended in a salt solution of Fries' minimal medium adjusted to pH 8 with NaOH. This procedure was repeated twice. The salt solution of Fries' minimal medium adjusted to pH 8 has been found to stop the reaction of alkylating compounds and NA with conidia immediately.

Standard conditions for treatment with NA, EMS, HA and ICR-70.

NA treatment: The conidio were suspended in 0.05 M sodium acetate buffer at pH 4.5. One volume of freshly prepared 0.02 M sodium nitrate solution was added to 3 volumes of conidiol suspension. The final concentration was 0.005 M $NaNO_2$, and the treatment was quenched as described above 40 minutes after the start of the treatment.

EMS treatment: The conidia were suspended in a 0.067 M phosphate buffer at pH 7.0. The treatment was started by adding enough EMS to bring the final concentration to 0.1 M; the treatment was quenched 300 minutes later.

HA treatment; Before the HA treatment the conidia were suspended in 3 M NaCl and then further diluted five times in the reaction mixture of Strack, et al. (1964 Mutation Res. 1: 10) which is composed of NH_2OHCl 2.6 g., H_2O 10 ml, 4 M NaCl 17 ml, and 10 M NaOH 2.5 ml, giving a pH of 6.2. The final HA concentration is 1M. Five minutes before the treatment was quenched, the conidio were centrifuged and decanted and at the quenching time, i.e. 300 minutes after the start of the treat-ment, the conidio were resuspended in 3 M NaCl. This washing procedure was repeated twice and then the conidio were suspend-ed in the salt solution of Fries' minimal medium adjusted to pH 8.

ICR-170 treatment: ICR-170 is the code number assigned to 2-methoxy-6-chloro-9-(3- ethyl-2-chlorethyl -aminopropyl-amino) ocridine dihydrochloride by H. J. Creech ond co-workers of the Institute for Cancer Research, Philadelphia. Forward mutations induced by ICR-170 in Neurospora hove been analyzed by Brockman ond Goben (1965 Science 147: 750). The conidio were suspended in a 0.067 M phosphate buffer at pH 7.0. The treatment was started by odding 1 volume of a freshly prepared solution of ICR-170 (250 mg/liter of water) to 49 volumes of the conidiol suspension, which gave a final concentration of 10.58 μM/ liter. The treatment was quenched as described above 130 minutes after the start of the treatment. The treatment and other manipulations involving ICR-170 ond conidio were performed under red light to eliminate the photodynamic effects associated with the ocridine ring. Plates were also incubated in the dark for at least 24 hours to allow sufficient time for the conidio to give rise to small colonies.

Plating medium. To estimate the viability of the treated and untreated conidio, they were plated in Westergaard's minimal medium supplemented with sorbose (15 g/liter), glucose (0.5 g/liter), fructose (0.5 g/liter), Casamino acids (200 mg/liter), a vitamin solution as in glycerol-complete medium (1 ml/liter) and adenine sulfate (25 mg/liter). To estimate the number of revertants the conidio were plated in the some substrate used for scoring survivors but supplemented with 0.2 mg adenine sulfate per liter instead of 25 mg/liter.

In the plates used to determine survival the density of the conidio was 5-10/ ml of substrate in a total volume of 100 ml. For scoring of revertants after NA, EMS or ICR-170 treatment, the conidio were plated to a density of 10^6 conidia/ml and 2 x 10^5 conidia/ml, each in a total volume of 100 ml. For scoring of the revertants after the HA treatment, the density of the conidio was 2 x 10^5/ml in a total volume of 500 ml. The plating was done in 15 x 200 mm intergrid phage dishes.

Statistical test. The test for significance is done according to Birnbaum (1954 J. Am. Stat. Assoc. 49: 254). In this test the number of revertants is considered as having a Poisson distribution. The probability is calculated by assuming that the

following two ratios belong to the same population:

(1) $$\frac{\text{Total population (surviving after treatment)}}{\text{Total population (untreated)} + \text{Total population (surviving after treatment)}}$$

(2) $$\frac{\text{Total number of revertants (in treated population)}}{\text{Total number of revertants (in untreated population)} + \text{Total number of revertants (in treated population)}}.$$

A probability lower than 5% indicates a significant difference between the number of reversions obtained in the control and in the treated series.

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