Henningson, K. W. Neurospora class experiments.

The following Neurospora class experiments are used in a course in biochemical genetics at the Institute of Genetics, University of Copenhagen.

Experiment 1. Tetrad analysis (ascospore color).

A cross between a mutant, asco (37402)a, with colorless ascospores (requirement for lysine) and a wild type strain, 74A, is used for tetrad analysis. Since the color of the ascospore is an autonomous character, it is possible to analyze the segregation of the alternative characters of the ascospores within the intact cell. The cross between strains of opposite mating types can be performed in two different ways: if the two strains are inoculated simultaneously, each strain acts as both female and male parent. If desired, one or the other strain can be used as female, by inoculating it before-hand on the crossing medium (Westergaard and Mitchell 1947 Am. J. Botany 34:573) to induce formation of protoperithecia. Then, mating of protoperithecia can be carried out with conidia from the other strain. Either solid or liquid medium may be used. In the case of liquid substrate, filter paper is used to support hyphae and protoperithecia.

Materials:

First day: Test tube cultures of 37402a and 74A. Three test tubes containing 10 ml liquid crossing medium + 300 mg/l lysine. 0.90% NaCl . Strips of filter paper (sterile).

Second day: 2% sodium hypochlorite. Distilled water. Microscope slides and coverslips. Petri dishes, needles and forceps for dissection.

Experimental procedure:

First day: Harvest conidia from both strains by pipetting about 3 ml of 0.9% NaCl into the test tube culture and shaking gently. Transfer from each strain 0.5 ml of spore suspension to the crossing medium. In each of the cultures a strip of filter paper is oriented so that about 5 cm stands out of the liquid. The cultures are incubated at 25C for 3-4 weeks.

Second day: Analysis of the tetrads. After about 3 weeks, the pyriform perithecia contain asci with maturing ascospores. The filter paper which carries most of the perithecia is removed from the test tube and placed in a petri dish containing 2% sodium hypochlorite. After 3-5 minutes, the conidia have been killed by the hypochlorite and the filter paper can be transferred to another dish and washed with distilled water. A few perithecia then are placed on a microscope slide . Under a dissecting microscope the contents of the perithecia are removed in a drop of distilled water and the perithecial wall is removed from the slide. A coverslip is placed on top and the asci are spread by a light pressure. Under the light microscope all asci containing 4 black and 4 colorless ascospores are analyzed. The number of first and second division segregation (pre- and post-reduction) tetrads is determined and the distance between the gene for ascospore color and the centromere is calculated from the formula: ½ x percentage of post-reduction tetrads.

Reference: Stadler 1956 Genetics 41:528.

Remarks: As the ascospores approach maturity, some asci containing 8 black spores may appear. If these abnormal asci are excluded from the results, the distance between the gene and the centromere is about 14%.

Preparing cultures: The asco strain (37402)a, FGSC#405, which requires lysine, is kept on Vogel's minimal medium + lysine 300 mg/l. Wild type strain 74A is kept on Vogel's minimal medium N (Vogel 1956 Microbial Genet. Bull. 13:42). Stock cultures are maintained and tested before use as described for arginine mutants.

Experiment 2. Isolation and characterization of biochemical mutants.

A. Isolation of biochemical mutants by filtration technique.

Conidia from a wild type strain of N. crassa are irradiated with X-rays and subjected to differential germination on liquid medium. Passage of the culture through a suitable filter at various intervals removes more and more of the germinated wild type conidia until finally the culture contains only conidia which are unable to germinate on minimal medium. After germination and isolation of these residual conidia on complete medium, many show specific growth-factor requirements.

Experimental procedure:

(1) Harvest conidia from 6 cultures of wild type strains 74A by pouring glass pearls into the flasks and shaking. Then pipette 10 ml 0.9% NaCl into each flask and shake again. Filter suspension through a G-2 glass filter. Centrifuge filtrate at 3000 x g for 3-5 minutes and resuspend pellet in 0.9% NaCl . Repeat 3 times. Determine the conidial concentration of the suspension by counting the spores in a haemocytometer. Adjust the final concentration to 1×10^8 spores/ml . A minimum of 10 ml of the final concentration is needed.

(2) Pipette 3 ml of the conidial suspension into each of 2 alcohol-sterilized plexiglass dishes that will fit into the X-ray machine. Irradiate one dish at 4×10^4 r (100 kv, 8 cm. distance, 1.7 mm aluminum filter) for 15 minutes. The second dish is kept as a non-irradiated control .

(3) Transfer the irradiated suspension to a test tube. Rinse the plexiglass dish with 3 ml 0.9% NaCl and add this to the test tube. Pipette 4 ml of this irradiated suspension into a 2-liter flask containing 400 ml Vogel's N-minimal medium + 0.5% sucrose. Prepare a control flask of the non-irradiated sample in the same manner. Place the flasks on a mechanical shaker and shake at low speed at 32C. Record inoculation time as zero time for growth.

(4) To determine the survival and germination percentages, dilute 1 ml of the remaining irradiated and control suspensions to 1,000 and 100 conidia/ml. Transfer 5 samples of 1 ml from each dilution to 5 tubes each containing 15 ml of complete medium with sorbose (melted and kept at 42C). Shake and pour into sterile petri plates. Incubate at 20-25C for 2-3 days (may be stored at 4C).

(5) To remove the germinated wild type spores, which have formed hyphae, filter the contents of the two flasks through 4 layers of cheesecloth which have been previously fixed in the necks of 2-liter Erlenmeyer flasks and sterilized in situ . After filtration, the filter is replaced by a sterile cotton plug and the flask is placed on the shaker. Ten filtrations are carried out, at 5, 8, 10, 12, 15, 18, 21, 24, 31, and 42 hours after inoculation, respectively. Platings are made from the 8th, 9th and 10th filtrations.

(6) Following the 8th and 9th filtrations, pipette 25 ml from each culture into a sterile flask and plate 20 aliquots from each sample as described above (if necessary the samples can be stored at 4C and plated the following day). From the last filtration, prepare as many plates as possible. All plates are incubated at 32C for 2 days. (May be stored at 4C until analysis can be carried out.)

(7) Determine the number of colonies and calculate the germination and the survival percentages. The number of viable conidia per ml in the cultures, when started, and following the filtrations, is a rough measure of the effectiveness of the filtration.

(8) Isolate colonies, mainly from the last filtration, in small tubes containing 2 ml of complete medium. For transferring small pieces of colonies, a sharp rigid inoculation needle is used. Incubate the cultures at 32C for about 7 days.

(9) Test the colonies isolated on complete medium for growth-factor requirements by transferring conidia to liquid minimal medium N with a loop-shaped inoculating needle. Keep the two cultures together at 32C for 2 days. Use the isolated colonies which are not able to grow on minimal for further analysis. Calculate the percentage of biochemical mutants.

References: Catcheside 1954 J. gen. Microbiol. 11:34; Fries 1947 Nature 150:199; Lester and Gross 1959 Science 129:572; Pontecorvo, Roper, Hemmons, MacDonald and Bufton 1953 Adv. Genet. 5:141; Woodward, De Zeeuw and Srb 1954 Proc. Natl. Acad. Sci. U.S. 40: 192.

Preparing cultures: Wild type strain 74A is kept on minimal medium N. Samples from stock cultures are spread on plates of medium N. The hyphae are used to inoculate agar slants. Conidia are spread on minimal crossing medium + sorbose and single colonies are used to inoculate agar slants of medium N. Then, conidia are used to inoculate six 100-ml Erlenmeyer flasks, each containing 20 ml of crossing medium + 2.5% glycerol (no sucrose). Incubate for 7 days at 20C.

B. Preliminary test of biochemical mutants.

To determine the type of growth-factor requirements of the biochemical mutants, preliminary tests are carried out on mixtures of growth factors. In this way mutants can be characterized as: a) amino acid-requiring, b) vitamin-requiring, or c) purine and pyrimidine requiring.

Materials:

One petri dish (17 cm in diameter) of each of the following media: synthetic crossing medium + sorbose; the same + sorbose + casamino acids; the same + sorbose plus a vitamin mixture; the same + sorbose + hydrolyzed yeast nucleic acids. 10 small tubes with complete medium N. 0.9%

NaCl and centrifuge tubes. 5 cultures of the biochemical mutants isolated in the filtration experiment. 5 cultures of biochemical mutants with known requirements.

Experimental procedure:

Prepare a suspension of conidia from each mutant by adding 2 ml of 0.9% NaCl to each tube and stirring briefly. Pipette the suspension into a centrifuge tube and adjust the volume to 10 ml with 0.9% NaCl. Centrifuge at 3000 x g for 3-5 minutes. Decant and resuspend conidia in 10 ml 0.9% NaCl. Repeat 2 times. The final volume should be about 5 ml. Prepare a new culture from each mutant on complete medium N. Using an inoculating loop, place a small drop of the conidial suspension of each mutant on each of the media listed above under materials. Before inoculation, mark all plates in such a way that 8 of the mutants are placed at the periphery and 2 in the center. Incubate the plates at 25C for 2 days (can be stored at 4C). Classify the mutants on the basis of their growth factor requirements.

C. Auxanographic test:

Prepare a suspension of conidia from each of the mutants and inoculate, as described above, plates with individual growth factors. Test the amino acid-requiring mutants on plates of minimal crossing medium + sorbose and supplemented, singly, with 150 mg/ml of the following amino acids: L-arginine HCl, L-leucine, lysine, methionine, phenylalanine and proline. Test the mutants requiring purines or pyrimidines on minimal crossing medium + sorbose +adenine sulfate 150 mg/ml. Test the vitamin-requiring mutants on minimal crossing medium + sorbose supplemented with pyridoxine 50 mg/ml and with Ca-pantothenate 50 mg/ml, separately. On a normal sized petri dish, 4 mutants can be tested. Inoculate close to the periphery of the dishes at previously marked positions. Incubate at 25C for 2 days. Many of the auxotrophic mutants isolated by the filtration technique described can be classified by this auxanographic test.

References: Emerson 1955 Vol. 2, pt . 2, p. 443 In Hoppe-Seyler and Thierfelder (ed.) Handbuch der physiologisch- und pathologisch-chemisches Analyse, 10th ed. Springer, Heidelberg; Horowitz 1950 Adv. Genet. 3:33; Pontecorvo 1949 J. gen. Microbiol. 3:122.

Strains used and preparation of cultures:

The following strains with known requirements are used: pro-1 (21863), FGSC#29; phen-1 (H3791), FGSC#504; me-8 (P53), FGSC#98; me-5 (9666), FGSC#140; arg-1 (B369), FGSC#324. Cultures are kept on complete medium. Stock cultures are maintained and tested as described for arginine mutants.

D. Precursor test of arginine mutants.

The final step in the physiological characterization of the biochemical mutants is the precursor test. A group of mutants with known requirements for arginine is used to demonstrate the intermediate steps in the biosynthesis of arginine in Neurospora.

Materials:

Cultures of 7 arginine mutants. Plates of minimal medium supplemented as described below. 0.9% NaCl. Centrifuge tubes.

Experimental procedure:

Prepare a suspension of conidia from each of the mutants by adding 2 ml of 0.9% NaCl to each culture tube. Stir briefly and pipette about 1 ml of the conidial suspension into a centrifuge tube. Adjust the volume to 10 ml with 0.9% NaCl. Centrifuge at 300 x 9 for 3-5 minutes. Decant and resuspend conidia in 10 ml 0.9% NaCl. Repeat 2 times. The final volume should be about 5 ml. With a loop a small drop of conidial suspension is placed on the following media:

minimal crossing medium + sorbose; the same + L-arginine 150 mg/l; the same + L- citrulline 150 mg/l; and the same + L-ornithine 150 mg/l. Mark the plates and inoculate at the indicated spots at the periphery. Incubate at 25C for 2 days.

Determine the block in the biosynthetic chain for each of the mutants from their growth patterns. Establish the sequence of the arginine biosynthetic pathway.

References: Srb, Fincham and Bonner 1950 Am. J. Botany 37: 533; Srb and Horowitz 1944 J. Biol. Chem. 154: 129; Vogel and Bonner 1954 Proc. Natl. Acad. Sci. U. S. 40:688.

Cultures and maintenance:

The following strains of arginine mutants should be used: arg-1 (36703T), FGSC#273; arg-1 (B369), FGSC#324; arg-1 (46004), FGSC#528; arg-5 (27947), FGSC#274; arg-5 (27974), FGSC#480; arg-3 (30300), FGSC#1068; and arg-3 (30300), FGSC#1069. The strains are kept on Vogel's minimal medium N +arginine 150 mg/l. Stock cultures are covered with sterile paraffin oil as soon as conidia are formed and are placed at 4C. Samples from a stock culture are taken with a loop and spread on plates. Hyphae formed on the plates are used to inoculate agar slants. Conidia should be tested on liquid minimal medium N for back mutations. If back mutation has occurred, conidia should be spread on minimal medium N + sorbose + arginine and single colonies re-isolated onto agar slants. Each culture must then be tested on liquid minimal medium. Incubate cultures at 25-32C.

Media:

l) Synthetic crossing medium (Westergaard and Mitchell 1947 Am. J. Botany 34:573). A 4x concentrated stock solution is prepared. After dilution sugar is added and the pH adjusted with NaOH.

2) Vogel's minimal medium N (Vogel 1956 Microbial Genet. Bull. 13:42). A 50x concentrated stock solution is prepared. After dilution sugar is added and the pH is adjusted with NaOH.

3) Complete medium N. To each liter of medium N add: yeast extract 2.5 g., malt extract 5 g., vitamin mixture 10 ml, hydrolyzed nucleic acid 2 ml, and casamino acids 15 ml.

4) Vitamin mixture. Dissolve the following in 1 liter of water: thiamine 100 mg, riboflavin 50 mg, pyridoxine 50 mg, Ca-pantothenate 220 mg, p-aminobenzoic acid 200 mg, nicotinamide 200 mg, choline HCl 129 mg, inositol 400 mg.

5) Hydrolyzed nucleic acid. Dissolve 1% yeast nucleic acid and 0.2% thymus nucleic acid in 1 N NaOH. Hydrolyze for 24 hours at 35C. Adjust to pH 6.5 and store at 4C.

6) "Synthetic casein" (casamino acids). In 687 ml of water, dissolve the following: glycine 20 mg, DL-alanine 180 mg, L-leucine 243 mg, DL-isoleucine 485 mg, DL-aspartic acid 410 mg, L-glutamic acid 1090 mg, DL-serine 580 mg, DL-threonine 390 mg, L-proline 400 mg, L-hydroxyproline 10 mg, L-cystine 15 mg, DL-methionine 360 mg, DL-valine 790 mg, DL-phenylalanine 390 mg, L-tryptophan 110 mg, L-tyrosine 325 mg, L-arginine HCl 305 mg, DL-lysine HCl 310 mg and L-histidine HCl 455 mg.

7) Sorbose-containing media. The ordinary carbon source, sucrose, is replaced by a mixture of sorbose, glucose and fructose to give final concentrations of 1.5%. 0.04% and 0.04%, respectively.

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