Selection of intramural-enzyme mutants

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Abstract
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In studies of enzymes induced by lactose and galactose in Neurospora, it has become apparent that growth conditions must be carefully controlled, and that shaker cultures containing a single carbon source can provide reproducible conditions well-suited for these studies. The ideal carbon source should cause no repressive interference with the induction process. Glycerol is suitable for such studies, but wild type strains are quite variable in their ability to grow on glycerol under the required conditions. The isolate 105-L5-A (formerly designated 55D, Bates and Woodward 1967 Neurospora News. 12:11) shows greatly improved growth on glycerol when compared with STA4. Cresser of this isolate to wild type 74-ORB-1a (Bates 1967 Genetics 56:543) yielded a variety of isolates with improved glycerol growth characteristics, although initial selection was for lactose growth. Two of these were crossed (211-L5-a x 341-8A) and an isolate designated 41-L5-A was obtained. This isolate has been used for all subsequent glycerol growth studies.

Growth conditions are: rotary shaking, 3/4 inch radius, 150 cycles per minute, 30 ± 0.5°C, 0.18 M glycerol, Vogel's medium, 200 ml in 500 ml Erlemeyer flasks, mounted at a 30 degree angle. The Vogel's medium is autoclaved at 2 x concentration, and the carbon source is autoclaved separately in 100 ml water. The inoculum is 106 conidio per ml medium. Under these conditions growth is linear for 90 hours (yielding ca. 0.15 g dry weight), and comparisons are made by harvesting at 48 hours (yielding ca. 0.7 g dry weight). The rate of growth with glucose under these conditions is ca. two times the rate obtained with glycerol.

In comparison with 411-L5-A, taken as 100%, growth of some wild type strains on glycerol can be grouped in the following way: STA4 and RL-A, 33-36%; ST73a, RL-o and Em-o (FGSC 691), 58-68%; Em-o (FGSC 692), 103% (all based upon total mycelial dry weight at 48 hrs). RL-A and RL-o are Rockefeller-Lindgren isolates obtained from J. F. Wilson. It is apparent that a mating type has a better glycerol growth than does Em-o for all three strains, except for 411-L5-A. When grown on sucrose under other identical conditions, little difference (± 10%) in total growth is observed among these different strains. Another distinguishing characteristic is the orange pigmentation which occurs under these glycerol growth conditions in an inverse relationship to ability to grow on glycerol. The Em-o and 411-L5-A cultures show no evidence of this pigmentation.

Among the isolates obtained along with 411-L5-A, there was a marked correlation between ability to grow on glycerol and reduced production of conidia. For example, 411-L5-A producer only 30-50% of the conidia produced by STA4 when grown and harvested under the same conditions. This characteristic is not necessarily associated with glycerol growth is shown by Em-o, which conidiatizes more abundantly than STA4, but which grows well on glycerol. Crosses designed to combine the glycerol growth characteristics with amino acid and inositol requirements are now in progress. The 411-L5-A isolate producer abundant protopersithecia on Westergaard's synthetic cross medium, and up to 90% spore viability, but the mature perithecium apparently have low internal pressure, and discharge ascospores weakly.

It appears that the 411-L5-A amino acid auxotrophs have very similar glycerol growth characteristics, but these strain have not been completed. Such characteristics would allow very precisely controlled study of incorporation of labeled amino acids during induction studies. If there are isolates which appear to be potentially useful to other workers, the set of cultures will be deposited in the Fungal Genetics Stock Center. (Supported by NSF Grant GB 5189). * Department of Biology, The Univ. of North Carolina at Greensboro, Greensboro, North Carolina 27412.

**Notes**

- Selection of intramural-enzyme mutants.
- Sargent, M. L. and H. D. Braymer.
- Metzler, G. and W. D. Metzenberg 1967 Arch. Biochem. Biophys. 120:487; i.e., that release is cytotoxic.
- Sargent, M. L. and W. D. Metzenberg were unable to detect cytotoxic release of glucose from invertase-hydrolyzed sucrose. However, their reconstitution experiments with the available invertase mutant (spontaneous) demonstrated that wild-type conidio suffered inositol-less death 10-100 times faster than the mutant conidio in mixed populations. This result coupled with our ability to select invertase and trehalase mutants suggests that the assumption is valid in certain situations.
We have attacked the problem of crossfeeding in five ways. First, parent strains with a minimum amount of enzyme were chosen. From these, partial mutants were obtained, and were used as parents for subsequent mutant hunts. Second, conidia were dialyzed in water to deplete endogenous reserves which might allow initial growth. Third, enzyme on the surfaces of conidia was inactivated by treatment with acid (Metzenberg 1963 Biochem. Biophys. Acta 77: 455). Fourth, inositol-less death selection on plates (Lester and Gross 1959 Science 120: 572) was chosen over filtration selection, since cell death occurs more promptly after germination with the inositol-less death technique. Fifth, selection was conducted, where practical, on a substrate which is hydrolyzed rapidly enough to support growth and inositol-less death, but slowly enough to prevent accumulation and crossfeeding of reaction products (e.g., glucose). We used raffinose as a carbon source for invertase mutant hunts, and low levels of trehalase in the trehalase mutant hunts.

Cultures of inositol-requiring strains (allele 89601) were grown for 7 days at 20°C on Vogel's salts, 1.5% sucrose, 20 μg/ml inositol, and 2% agar. Conidia were harvested in water, filtered through cheesecloth and glasswool, and dialyzed against three changes of distilled water (once overnight). The washed conidia were collected by centrifugation, and resuspended in water (107 conidia/ml). An equal volume of 0.2 N HCl was added and the suspension was shaken at 37°C (8 min. treatment wholly inactivated invertase and trehalase with >40-60% survival of cells). The acid treatment was terminated by bringing the suspension to pH 5.5 with 1.0 M sodium citrate. The acid-treated conidia were collected by centrifugation (60-80% recovery), resuspended in 0.1 M sodium citrate, pH 5.0 (1.2 x 10^7 conidia/ml), and either irradiated with UV light (to 10% survival) or shaken at 25°C with an equal volume of 50 μg/ml N-methyl-N'-nitro-N-nitroso guanidine (MNNG) made up in the same pH 5.0 buffer (15 min. treatment, 40-60% survival). MNNG was more efficient in producing the desired mutations, but increased both the reversion rate at the inositol locus, and the number of secondary mutations in the selected strains. The mutagenized conidia were collected by centrifugation, washed, resuspended (1.5 x 10^6 conidia/ml) in Vogel's salts and a carbon source (trehalose or raffinose, 0.1%), and incubated for 6 hours at 25°C. After this incubation, the conidia were filtered through cheesecloth and glasswool, collected, washed twice with water, and resuspended in water (1.06 conidia/ml). One ml aliquots were spread on prepared plates (Vogel's salts, 0.2% carbon source and 1.5% agar). After 3-5 days incubation at 25°C, the plates were overplated with Vogel's salts, 150 μg/ml inositol, 1.5% fructose, 3.0% sorbose, and 1.5% agar, and incubated at 25°C.

The colonies which appeared were either isolated into separate slants for subsequent analysis (trehalase mutant hunts), or scored directly on the plates with a liquid, in vivo, invertase assay. The assay solution is prepared by dissolving 500 mg glucostat enzyme reagent (Worthington Biochemical Co.) in 45 ml of 44% ethanol, adding 42 mg tartrazine and 100 mg o-tolidine, filtering and adjusting the pH to 5.0 with 5% formic acid. Immediately before use the above solution is diluted 1:1 with 1.0 M sucrose. After adding 3-4 ml of the combined solution to each plate, and allowing it to soak into the agar for 20-30 min., the residue is poured off. Within 30-60 min. all colonies possessing invertase activity are surrounded by a bright green halo, representing the liberated glucose, while mutant colonies remain white with no halo. This reaction is light-sensitive, and is best done in subdued light.

While none of these techniques are particularly novel, their combined use has allowed us to isolate previously unobtainable mutants. Over 200 trehalase mutants (many with no detectable activity) have been isolated at the end of three cycles of experiments, in which the best partial mutants recovered in one were used as parents in the next. In addition, two cycles of selection have yielded five partial invertase mutants (having less than 10% of the invertase activity of the original parent). We hope additional cycles will produce complete invertase mutants similar to the one known spontaneous mutant (Sargent and Woodward 1969 J. Bact., in press). The genetics and biochemistry of the trehalose mutants will be reported elsewhere. We thank D. O. Woodward, A. S. Sussman, and R. H. Davis for advice in the course of this work. - Department of Botany, University of Illinois, Urbana, Illinois 61801, and Department of Microbiology, Louisiana State University, Baton Rouge, Louisiana 70803.