Ureaseless mutants in Neurospora crassa

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Abstract
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both resistant and sensitive strains was enhanced when hydrolyzed yeast nucleic acid was added to the acriflavine minimal medium. The double mutant acr-2<sup>-r</sup>;acr-3<sup>-r</sup> was more resistant than either single mutant. In scoring acriflavine resistance, false negatives may result if inocula are too small.

acr-rr appeared to be recessive, while both acr-2<sup>-r</sup> and acr-3<sup>-r</sup> were dominant. This was inferred from the observation that at some concentrations, the amount of growth of acr-1<sup>-r</sup>+acr-1<sup>-r</sup> heterocaryons was close to that of the wild type, while growth of acr-2<sup>-r</sup>+acr-2<sup>-r</sup>, or acr-3<sup>-r</sup>+acr-3<sup>-r</sup> heterocaryons was close to the mutant type. The dominance of acr-2<sup>-r</sup> and acr-3<sup>-r</sup> was in agreement with the fact that at least five of the seven mutants isolated from the macroconidial strains were heterocaryotic for the mutant alleles. Such heterocaryons would probably not be able to survive the inhibitory effect of acriflavine if the mutant alleles were recessives.

All acr-2 mutants were cross-resistant to 3-amino-1,2,4-triazole, and all acr-3 mutants, to malachite green. The double mutant acr-2<sup>-r</sup>;acr-3<sup>-r</sup> could grow in the presence of either chemical. The concentrations at which resistance and sensitivity could be differentiated were 0.25% in slants and 0.1% in liquid culture for 3-amino-1,2,4-triazole, and 2 μg/ml in slants and 0.5 μg/ml in liquid for malachite green. These mutants did not differ appreciably from wild type in their sensitivity to acridine orange, profazine, thionine, and crystal violet.

Mutations from acriflavine resistance to sensitivity seem to be frequent. Mutants at either acr-2 or acr-3 have been recovered from most of the strains upon first testing when between 10<sup>5</sup> and 10<sup>7</sup> conidia from each strain were plated on the acriflavine plates. Mutants resistant to the dye have also been obtained by Howe and Terry (1962 Can. J. Genet. Cytol. 4: 447) and by M. E. Case (personal communication), Department of Biological Sciences, Stanford University, Stanford, California.

**Jho, K. K.**  Indole excretion by revertants derived from indole-accumulating trp-3 (td) mutants.

Indole is known to be excreted by many trp-3 mutants. Accumulation of indole in the medium is a consequence of the ability of the mutant protein to carry out the reaction: indole glycerol phosphates indole + triose phosphate (DeMoss and Bonner 1959 Proc. Natl. Acad. Sci. U. S. 45: 1405).

It seems that at least some of the prototrophic revertant trp-3 mutants also accumulate indole in the medium. A large number of phenotypic revertants from three trp-3 mutants (9A-2, td-71 and 9A-100) were isolated from UV-treated conidia by plating them on indole-supplemented minimal medium. The object was to isolate, if possible, some revertant strains which were still auxotrophic but could grow on either indole- or tryptophan-supplemented medium. Though such strains were not found, it was noticed that most of the prototrophic strains accumulated indole in the medium. The indole-accumulating revertants were four to seven times more frequent than the non-accumulating ones. Indole accumulation by a majority of isolates was expected because of the multiple, heterocaryotic nature of the conidio which gave rise to these strains. But the possibility was considered that the accumulation of indole was on inherent characteristic of the revertant nuclei.

Two of the indole-accumulating, fat-growing isolates (to avoid possible suppressed strains) were crossed to the closely-linked marker "fluffy" strain. Conidial, non-fluffy random ascospore isolates from each cross were found to contain both auxotrophs and prototrophs (23 prototrophs among 60 conidial isolates in one case and 76 prototrophs among 92 isolates in the second cross). In both cores, with the exception of four isolates in the second cross (out of 75 isolates), all of the prototrophic, conidial isolates were found to accumulate indole in the culture medium; the four non-accumulating isolates could, presumably, represent cross-over products between the trp-3 and tr loci. Accumulation of indole by the isolates was quantitatively comparable to that of their parent auxotrophs.

These results indicate that the enzyme tryptophan synthetase in these revertants may be different from the wild-type enzyme. DeMoss (1962 Biochem. Biophys. Acta 62: 279) has shown that the wild-type enzyme does not permit accumulation of free indole in the synthesis of tryptophan from indole glycerol phosphate. Esser et al. (1960 Z. Vererbungslehre 91: 219) have shown that about half of the revertants from the indole-accumulating strain td-2<sup>-r</sup> had a tryptophan synthetase which could be distinguished from the wild-type enzyme by enzymatic and immunologic criteria. It was not reported whether or not some of the revertants accumulated indole.

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**Kolmark, H. G.** Ureaseless mutants in Neurospora crassa.

The presence of the enzyme urease in Neurospora has been known for many years (Srb and Horowitz 1944 J. Biol. Chem. 154: 129). Mutation to an ureaseless condition is therefore to be expected.

In the present experiments it was first found that urease could very easily be demonstrated directly on a growing colony, simply by rooking a small piece of pH-indicator paper (range about pH 6-8) in on 8% solution of urea in water and placing it in contact with the mycelium or conidia. A color change towards the alkaline takes place in a few minutes due to liberation of ammonia when urea is enzymatically hydrolysed.

This change in pH was utilized in the screening methods finally worked out for isolation of ureaseless mutants.

Strains of Neurospora: Some earlier experiments with macroconidial strains of colonial morphology did not succeed in the isolation of ureaseless mutants. A microconidial strain, 398-28 A, was then tried in the expectation that mutants might more easily become phenotypically expressed when induced in mononuclear conidia. A morphological mutant, m-25, derived from this strain after treatment with ultraviolet light, was used in some of the later experiments. m-25 forms very small and extremely dense colonies.
Experimental Procedures: Suspensions of conidio in saline were irradiated with UV and, after appropriate dilutions, incubated in 25 ml of liquid minimal medium in petri dishes kept at 21-22°C. Under these conditions the colonies grow below the surface and adhere to the gloss bottom of the plate. After 6-7 days they are ready to be tested for urease activity.

The medium is then decanted off. With careful handling the colonies stick to their growth place on the gloss. They are rinsed twice with saline. After the last rinsing the plates or placed in a slanted position for a few minutes to let superficial saline drain off. A filter paper is moistened with a mixture of 4% (or stronger) urea and a pH indicator (brom cresol purple or brom thymol blue) in the range 5.5-7.5, adjusted to the lower pH with a weak phosphate buffer (ionic strength 0.01). When the paper is pressed against the colonies with a gloss plate, all normal colonies changes the indicator color in a few minutes.

Non-reacting colonies are isolated into tubes with minimal medium. Established cultures are restested later in comparison with the original strain.

In an alternative procedure, the UV-treated conidio or spread on the surface of solid medium. A white net of nylon fabric, 0.3 threads per mm, is placed on top of the inoculated medium. The colonies, appearing between the meshes, are after 3 days overlaid with 15 ml of liquid minimal medium and th. incubation continued for 5 days more. The liquid medium is then decanted off, with careful handling the colonies stick to their growth place on the plate after 6-7 days they are ready to be tested for urease activity.

Isolations or. mod. from the untested part of the colonies were left on the plate.

Two cultures out of a total of 58 isolates were by retesting found to be entirely lacking in urease activity, as evidenced by no shift towards a positive reaction during 4-5 days. The ureaseless mutants show a positive reaction when a small amount of mycelium was incubated at 34°C in 0.25 ml of 4% urea, dissolved in 0.01 ionic strength phosphate buffer. The reaction is started at pH 6 with the indicator brom thymol blue. Th. original strain gives a strongly positive reaction in 2 hours.

Several other isolates appear to have a decreased urease activity. The frequency of ureaseless mutants was found to be about 1 per 10^4 survivors. The ureaseless mutants or. unable to grow on urea as the sole source of nitrogen, while the original strain grows well. Otherwise, the mutants show no decreased vitality with regard to germination of conidio and ascospores nor in mycelial growth under the conditions observed.

Genetic testing: On. of th. ureaseless mutants, u-9 A, was crossed to th. wild type strain 74-ORB-la. The ureaseless character was found to segregate regularly at 1 or 2 nd linkage group and only 2 linkage group were in progress, but so far it can only be stated that it seems not to be situated in the linkage groups I, II or VII. It is proposed that the mutant phenotype described above be designated by the symbol u, since this symbol seems not previously to be in use as a locus symbol in Neurospora.

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Motile, P. Elucidation of "inositol-less death." The phenomenon of "inositol-less death" in Neurospora is the basis of an efficient and widely used method for the isolation of heterotrophic mutants (Lester and Gross 1959 Scien. 129:572). Abnormal growth (Beadle 1944 J. Biol. Chem. 156:683) and Myloie Nature 171:179) of suboptimally cultured inositol-less strains has been explained by an unbalance between the synthesis of and other cellular constituents (Shatkin and Tatum 1961 Amer. J. Botany 48:760). Still, the peculiarity of inositol-less mutants remains mysterious.

An investigation of the mechanism of the utilization of exogenous proteins in Neurospora has led to the detection of a constitutive cytoplasmic particle which contains the proteolytic enzymes to be secreted into protein-containing growth media (Motile 1965 Z. Zellforsch. 65:884). These secretory granules have been termed protease particles (Motile 1964 Naturwissenschaften 51:489) they represent small spheres (diameters from 0.15 to 0.3u) surrounded by a single membrane (Motile et al. 1965 Z. Zellforsch, in press). Incorporation of either choline-C14 or inositol-C14 into respective heterostrophic strains followed by cell fractionation (density gradient centrifugation) and analysis of the lipids has shown that the composition of th. membranes of protease particles is significantly different from that of other cytoplasmic membranes: they are relatively poor in lecinthin and rich in inositol phospholipid.

If on inositol-less strain is cultured at a high level of exogenous inositol (50 µg/ml), th. proteolytic activity is concentrated in the fraction which contains th. protease particles. However, at a suboptimal level of inositol (0.5 µg/ml) the protease activity is contained mainly in th. soluble fraction, only a small percentage still being located in th. position when the protease particles or. normally found in the density gradients.

There findings lead to th. conclusion that a shortage of inositol results in insufficiently tightened protease particles and subsequent release of proteases into th. cytoplasm. Since in homogenates from woprimotically cultured mycelia incubated at 28°C a much more rapid breakdown of protein occurs than in extracts from normally grown mold, it seems to be very likely th. fr. fraction of protease particles is not in th. cytoplasm. In germinating conidio cultured in the absence of inositol, the autolysis may become complete due to the absence of septa and germ tubes. At low concentrations of inositol, growth of the hypha proceeds unless the inositol is exhausted. In this co., degradation may affect only that part of th. hypha which has been formed (probably th. tip); since the autolysis of cytoplasm results in the liberation of fr. inositol (Fuller and Tatum 1956 Amer. J. Botany 43:361) a further limited growth of th. surviving part of th. hypha will take place, the repithet of this process leading to a high number of highly branched small colony.

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