Enrichment of Auxotrophic Mutants of *Aspergillus flavus* by Tritium Suicide

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A method based on tritium suicide was developed to enrich auxotrophic mutants of *Aspergillus flavus*. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was chosen as a mutagen, since a wide variety of mutations were induced by the action of 0.1% NG on *A. flavus* conidia suspended in phosphate buffer (pH 7.0). The decimal reduction time under these conditions was about 30 min, and the surviving population contained 4 to 6% auxotrophs after 1 hr of mutagenesis. This proportion was then increased by tritium suicide of wild-type cells. At a concentration of 1.3 μ M, 3 H-leucine was incorporated better than 3 H-proline or 3 H-thymidine into the germinating conidia. With about 20 hr of incubation and a short treatment in a high-speed mixer to disentangle mycelia and conidia, a 5- to 20-fold decrease in the number of survivors resulted from the incorporated 3 H-leucine (5 c/mmole) after 1 week of storage at 5 C. At a 10-fold lower concentration, the uptake of radioactivity and the subsequent suicide rate were much lower. With 3 H-leucine, the proportion of auxotrophs in the surviving population rose from 5 to about 20% during 2 weeks of storage at 5 C. Mutants requiring various intermediates for protein or nucleic acid synthesis or requiring vitamins were isolated. Finally, it was noted that *A. flavus* shows a much higher resistance to tritium suicide than does *Escherichia coli*.

Investigations of the biosynthesis of aflatoxins (13) prompted our finding of an efficient method for the isolation of mutants of *Aspergillus flavus*. Because the conidia and mycelia of *A. flavus* were found to be relatively resistant to ultraviolet light (14) and to ethyl methane sulfonate (*unpublished data*), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), known to be a very effective mutagen for bacteria (1, 11), was tried. Furthermore, it seemed desirable to increase the percentage of auxotrophic mutants in the population after mutagenesis by a suitable enrichment technique. Methods available for this purpose include filtration (4) and several starvation techniques based upon the fact that certain single auxotrophs die more rapidly in a minimal medium than do double auxotrophs (5, 8, 16). In addition to the difficulties associated with the filtration technique itself (21), we observed that the ungerminated conidia of *A. flavus* became trapped in the entangling mycelia in shaken liquid cultures. The starvation technique requires that suitable auxotrophs be available to serve as the parent strain from which the desired double auxotrophs can be obtained. In the present instance, such single auxotrophs were not available.

It seemed reasonable, however, to expect that the tritium suicide technique proposed by Lubin (9) for the efficient isolation of auxotrophic mutants of cells of diverse origin would also be suitable for fungi. This report describes the adaptation of this method to *A. flavus*.

MATERIALS AND METHODS

Organism. An aflatoxin-producing strain of *A. flavus* (ATCC 15517) was used throughout this study and was maintained on Mycological Agar (Difco). Periodically, a batch of conidia was prepared by inoculating Mycological Agar in a Roux bottle with soil from a soil culture of *A. flavus* (12). After 1 week of incubation at 30 C, the conidia were washed from the agar surface with an aqueous solution of 0.1% (w/v) sodium lauryl sulfate. These conidia were then washed on an HA filter (Millipore Corp., Bedford, Mass.) with 0.01% (w/v) sodium lauryl sulfate, resuspended in 0.01% sodium lauryl sulfate, and stored at 5 C.

Viable counts were made by mixing an appropriately diluted suspension of organisms with a thin layer of melted Mycological Agar and incubating the mixture for 1 to 2 days at 30 C. Before plating, the suspension was violently mixed on a test-tube shaker. Such determinations yield only an approximate count of the total number of organisms in a suspension

because many conidia occur in chains, whereas hyphae tend to aggregate. Plate counts are therefore reported as the number of colony-forming units (CFU).

Media. The basal medium used for the incorporation of tritiated compounds contained (per liter): glucose, 50 g; $(\text{NH}_4)_2\text{SO}_4$, 3 g; KH_2PO_4 , 10 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; and sodium deoxycholate, 0.5 g. To 15 ml of this medium, we added ^3H -thymidine (6 c/mmole) or L-proline-3,4- ^3H (5 c/mmole) or L-leucine-4,5- ^3H (5 c/mmole) in quantities of 0.01 or 0.1 mc, depending upon the experiment. The pH was adjusted to 6.0 before sterilization by membrane filtration (Millipore Corp.).

The composition of the media used for identification of auxotrophs is given in Table 1. Glucose, Casamino Acids, tryptone, and yeast extract were added just before the media were autoclaved at 121 C for 15 min. A 100-fold concentrated vitamin solution (7) was sterilized separately by membrane filtration (Millipore Corp.) and was added later.

Mutagenesis. About 10^7 conidia were inoculated into 10 ml of 0.05 M potassium phosphate buffer (pH 7.0) and were incubated at 30 C on a rotary shaker bath. To this, we added 0.1 ml of acetone containing 10 mg of NG. At various times, 0.1-ml samples of the suspension were transferred into 10 ml of 0.1% (w/v) aqueous cysteine to inactivate the mutagen (6). After appropriate further dilutions, plate counts were made on C agar (see Table 1) to determine the rate of killing. After 1 hr of mutagenesis, the conidial suspension was filtered through an HA filter (Millipore Corp.), and the conidia were washed with 30 ml of 0.1% cysteine solution. These conidia were then suspended in a few milliliters of 0.01% sodium lauryl sulfate, spread on C agar in a Roux bottle, and incubated at 30 C for 1 week. A suspension of genetically heterogeneous conidia was then prepared as described above. This suspension was used in the subsequent studies of the enrichment of auxotrophic mutants by tritium suicide.

Isolation of auxotrophic mutants. From the suspension of genetically heterogeneous conidia (see above), a sample containing 10 to 20 CFU was plated on C agar in a petri dish. After growth and conidia forma-

tion at 30 C, conidia from isolated colonies were replicated with a needle onto M and C agar. About 10 isolates were tested per plate. Isolates growing on C agar that showed little or no growth on M agar were then replicated on V, A, Y, and C agar. Mutants that grew on A agar were screened for an amino acid requirement by auxanography. Conidia of such an isolate were spread with a few drops of water on M agar in a petri dish. After drying the agar surface for 3 hr at 30 C, small amounts of six mixtures of amino acids (7) were placed on the agar and the plates were incubated at 30 C. When a growth response to such a mixture occurred, the specific requirement was determined by auxanography with single amino acids. Those isolates that grew on Y agar but not on V or A agar were tested for growth on M agar by auxanography with added nucleic acid precursors (7).

Tritium suicide. After inoculation of 10^4 to 10^5 conidia into 15 ml of medium containing the tritiated compound (see above) in a 50-ml indented flask (Bellco Glass, Inc., Vineland, N.J.), the culture was incubated on a rotary shaker bath at 30 C. At various times, duplicate 0.1-ml samples were transferred into 0.9 ml of water and were filtered through an HA filter (Millipore Corp.) to remove the organisms. The radioactivity remaining in the medium was then determined by counting 0.1 ml of this filtrate in 15 ml of scintillation fluid on a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The scintillation fluid consisted of: dioxan, 1.5 liters; xylene, 0.5 liter; ethylene glycol monomethylether, 1.5 liters; naphthalene, 280 g; 2,5-diphenyloxazole, 35 g; and *p*-bis(2-(5-phenyloxazolyl))-benzene, 1.75 g. The counting efficiency was 11%. The counts reported have been corrected for evaporation losses of medium during incubation. After incubation, the culture was transferred with 10 ml of water into the 50-ml stainless-steel chamber of a Sorvall Omnimixer and was treated for 15 sec at top speed to disentangle hyphae and conidia. Samples were then diluted at least 10-fold to counteract reaggregation of the hyphae and were kept at 5 C. The killing effect of the incorporated radioactivity and the percentage of auxotrophs in the surviving population were then determined at various times as described above.

Chemicals. Mycological Agar, Vitamine Free Casamino Acids, tryptone, yeast extract, and agar (purified) were Difco products. NG was obtained from K & K Laboratories, Inc., Plainview, N.Y. ^3H -thymidine was obtained from Schwartz Bio Research, Inc., Orangeburg, N.Y.; L-proline-3,4- ^3H and L-leucine-4,5- ^3H were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Figure 1 shows the method developed for the enrichment of auxotrophic mutants of *A. flavus* by tritium suicide. Each step will now be discussed in some detail.

Mutagenesis. The lethal action of 0.1% (w/v) NG on *A. flavus* conidia could be expressed by first-order kinetics with a decimal reduction time

TABLE 1. Composition of media used for the preliminary identification of auxotrophic mutants (in grams per liter of deionized water)^a

Component	M agar	V agar	A agar	Y agar	C agar
Glucose.....	10	10	10	10	10
$(\text{NH}_4)_2\text{SO}_4$	4	4	4	4	4
KH_2PO_4	4	4	4	4	4
Vitamin solution (7).....		10			10
Vitamin Free Casamino Acids.....			2		2
Tryptone.....			2		2
Yeast extract.....				4	4
Agar (purified).....	15	15	15	15	15

^a The pH of each medium before sterilization was 6.0.

FIG. 1. Method for the enrichment of auxotrophic mutants of *A. flavus* by tritium suicide.

of about 30 min. NG is also a powerful inducer of mutations since, after 1 hr of mutagenesis at pH 7.0, auxotrophic mutants were consistently found to comprise 4 to 6% of the survivors. About 75% of the auxotrophs could be rapidly classified with the methods used. Requirements for 14 different intermediates in protein and nucleic acid synthesis and for various vitamins were found. Recognition of auxotrophic mutants by replica plating (17) was not possible because the wild-type organisms grew and sporulated more rapidly than most mutants. Incorporation of 2.0% sorbose in C agar, as practiced with *Neurospora crassa* (19), did not induce compact colonies. As many other non-nutritional mutations were observed, NG is clearly a potent mutagen for the conidia of *A. flavus*.

Uptake of tritiated compounds and subsequent suicide. The uptake of 0.1 mc of tritiated leucine, proline, or thymidine by germinating conidia is shown in Fig. 2. More leucine was taken up than proline or thymidine and this resulted in a more

rapid rate of death (Fig. 3). For good results, it was necessary to use at least 0.1 mc of the tritiated amino acid (the concentration is then 1.3 μM), even though only a part of it is taken up within 24 hr. At a 10-fold lower concentration, the absolute uptake of the three compounds per germinating organism was much lower, pre-

FIG. 2. Uptake of 0.1 mc of tritiated thymidine, proline, or leucine by 5×10^4 *A. flavus* conidia suspended in 15 ml of basal medium with the tritium compound.

FIG. 3. Survival of *A. flavus* at 5 C after incubation with 0.1 mc of the indicated tritium compound for 24 hr.

TABLE 2. Percentage of auxotrophic mutants in the surviving population during cold storage after incubation of a mixed population of *A. flavus* with tritiated leucine (0.1 mc)

Expt	Storage time (days)	Per cent survival	Isolates tested	Auxotrophs found	Per cent auxotrophs
1	0	100	210	10	4.7
	7	12	125	16	13
	15	0.8	51	13	25
2	0	100	105	6	5.7
	6	10	32	6	19
	16	1.6	24	3	12

sumably because of a reduced inhibition of endogenous synthesis. The subsequent rate of suicide was then also decreased (*unpublished data*).

The presence of sodium deoxycholate in the medium (3) and the short treatment in the high-speed blender minimized the undesirable tendency of hyphae and conidia to aggregate. This treatment did not affect the viability of the germinated conidia.

Enrichment of auxotrophic mutants. The results of two experiments in which 0.1 mc of tritiated leucine was used for suicide are shown in Table 2. It is apparent that the increase in the percentage of auxotrophs during cold storage resulted from the incorporated radioactivity. Nearly all of the mutants that were found before the suicide treatment could be reisolated afterwards during the cold storage period. A few mutants that were not found before the treatment were also isolated. The possibility exists that new mutants are induced during storage as a result of the tritium decay (15). We did not observe any enrichment when the tritium source was deleted from the medium.

DISCUSSION

NG is clearly an efficient mutagen for conidia of *A. flavus*. Recently, the application of NG to *A. nidulans* was described (2, 18); it appears, therefore, that NG is more suitable as a mutagen for molds than other agents previously used (4, 16, 20).

After mutagenesis, tritium suicide can be used to increase further the percentage of auxotrophs. For good results, the uptake of tritium per organism has to be sufficiently high to lead to rapid suicide. The compound to be used must not only have a high specific radioactivity, but also must be present in the medium at a level capable of preventing major dilution inside the organism as the result of endogenous synthesis.

At a concentration of 1.3 μ M, leucine gave better results than proline or thymidine. These results agree with those obtained by Lubin with *Escherichia coli* (10). It is entirely possible that a higher concentration of leucine than that which we used would lead to more striking results. Microscopic observation during the incubation with the tritium compound showed that some auxotrophs germinate slowly in the minimal medium. However, these mutants take up much less radioactivity than the rapidly growing wild-type mycelia and, during subsequent cold storage, they still become enriched. Contrary to results obtained with the starvation technique and *A. nidulans* (16), we did not observe a strong preferential enrichment of certain mutants.

Although the uptake of radioactivity was high (about 10^{-3} μ C/CFU or about 40 decays per sec per CFU), the observed rate of suicide was lower than that observed with *E. coli* cells which had taken up 100- to 10,000-fold less tritium per organism (9, 10, 15).

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