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Isolation of *Neurospora crassa* Bradytrophs

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A method was developed for the isolation of *Neurospora* bradytrophs. The bradytrophs (representing lesions in a number of pathways) were resistant to DL-*p*-fluorophenylalanine when growing in a leaky fashion but were sensitive when grown in the presence of their stimulating supplement.

Bradytrophs have been utilized for the study of many different kinds of systems in a variety of organisms (e.g., 1, 3, 5, 9). Most of the bradytrophs have been isolated as a minority class in procedures designed for the isolation of auxotrophs; however, the very nature of bradytrophs insures that they will be inefficiently recovered by most enrichment procedures simply because the leaky growth will be sufficient to allow effective counterselection as if they were wild types. I have developed an enrichment procedure for the isolation of a class of *Neurospora* mutants that are characterized both by bradytrophic growth and by resistance to DL-*p*-fluorophenylalanine (FPA) when they exhibit this leaky growth. These mutants are predominantly, although not exclusively, amino acid bradytrophs.

A number of variations on the same general theme were successfully employed. The technique that proved most useful is outlined here.

Conidia from approximately 2-week-old wild-type cultures (I have used both 74A and Emerson A) were plated (10^7 conidia per plate) in molten (45°C) Vogel minimal agar (13) with 1.5% sorbose, 0.2% glucose, and 0.2% glycerol (VSGG). After the agar had set, dry, sterile, glass fiber disks (Reeve Angel 934 AH) containing 0.2 mg of FPA were placed on the plates, which were then incubated at 33°C. Within 2 to 3 days, a dense mycelial lawn developed around a clear zone of inhibition (ZI). Within 4 to 5 days, colonies had developed throughout the ZI. Figure 1 shows a typical plate at this stage. The large colonies that developed near the center were usually conventional FPA-resistant mutants; however, the smaller colonies near the edge of the ZI often proved to be bradytrophs. Two such colonies are identified in Fig. 1. One was stimulated by arginine; the other was stimulated by adenine.

Table 1 shows the results of five separate experiments in which bradytrophs were isolated. In the first three, only colonies at the periphery were picked. In the last two, all colonies were picked regardless of location. The yield of bradytrophs varied from experiment to experiment; however, as these cells were not treated with a mutagen, considerable variation would be expected due to the chance preexistence of clones of bradytrophs in the wild-type stocks. It is possible of course that FPA acts as both a selective agent and a mutagen (7, 11, 12); however, according to B. J. Kilbey (cited in reference 6) and Baylis (2), FPA is not mutagenic for *Neurospora*.

Generally speaking, growth of the bradytrophs was stimulated by a single amino acid in each case. However, growth of some mutants was stimulated by either of two amino acids, by adenine, or by yeast extract, but by no single supplement or combination of specific supplements tested (combinations of supplements tested were those of reference 4, appendix E). Mutants were isolated whose growth was stimulated by glutamine, arginine, adenine, isoleucine, leucine, serine, methionine, tryptophan, histidine, homoserine, lysine, asparagine, proline, or tyrosine. In addition, one mutant was stimulated by either ornithine or proline, and one was stimulated by either phenylalanine or leucine.

Two possibilities were entertained to explain the growth of the bradytrophs in the ZI. Either the bradytrophs were inherently resistant to FPA or, by growing slower than the normal population, they survived until the FPA had been effectively removed by the killed wild-type cells in the ZI. (It was thought that the latter could occur either by irreversible uptake of the FPA or by dilution of the FPA with other amino acids that compete for uptake.) A simple test for the continuing presence of FPA was provided by heat killing the cells on a typical FPA disk plate after the ZI had been well established (3 to 4 days at 33°C). Suspensions of wild-type conidia were then streaked from the outside edge to the center of such plates, which were then reincubated at 33°C for 3 to 4 days. In every case, the

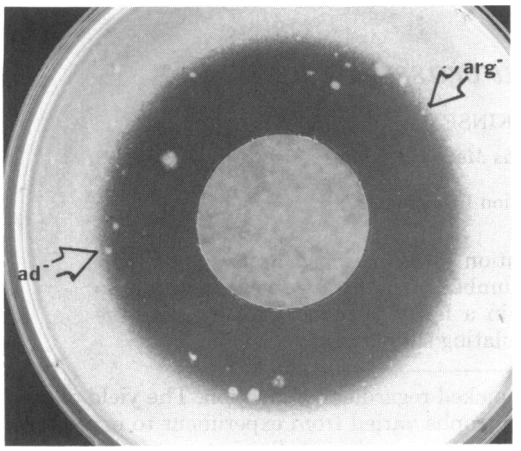

FIG. 1. *FPA disk bradytroph isolation plate (experiment 4 of Table 1). Twenty-three colonies were picked from the plate. The two bradytrophs isolated are identified.*

TABLE 1. *Isolation of bradytrophs*

Expt no.	Location of colonies picked	No. of colonies picked	No. of bradytrophs	Yield of bradytrophs (%)
1	Periphery of the ZI	20	5	25
2	Periphery of the ZI	35	21	60
3	Periphery of the ZI	19	3	16
4	Throughout the ZI	23	2	9
5	Throughout the ZI	172	28	16

streaks of developing mycelia showed vigorous growth at the outside edge of the plate; this growth ceased exactly at the edge of the previous ZI. Obviously, FPA was still present in a lethal concentration. Consequently, the bradytrophs which grew in the ZI were somehow resistant to FPA.

It seemed possible either that the bradytrophs were inherently resistant to FPA, or that, as in the effect of methyl mercury on *met-15* mutants of yeast (10), FPA at some critical concentration relieved the growth requirement of the mutants. To test these ideas, use was made of the double-gradient plating technique of Singh and Sherman (10). Plates of VSGG were prepared containing conidia (10^7 per plate) of either representative bradytrophic mutant strains, the wild type (74A), or auxotrophic mutant strains. Uniform strips of dry Whatman no. 1 filter paper, which contained 0.1 mg of FPA or 0.3 mg of the amino acid supplement that supported or stimulated growth, were placed in a crossed pattern on the plates. Each plate received one strip of FPA-containing paper and one containing the amino acid. The plates were incubated at 33°C for 3 days. Figure 2 shows the two basic growth patterns observed for the majority of the tested bradytrophs as well as the pattern observed for the wild type and a histidine-1 auxotroph.

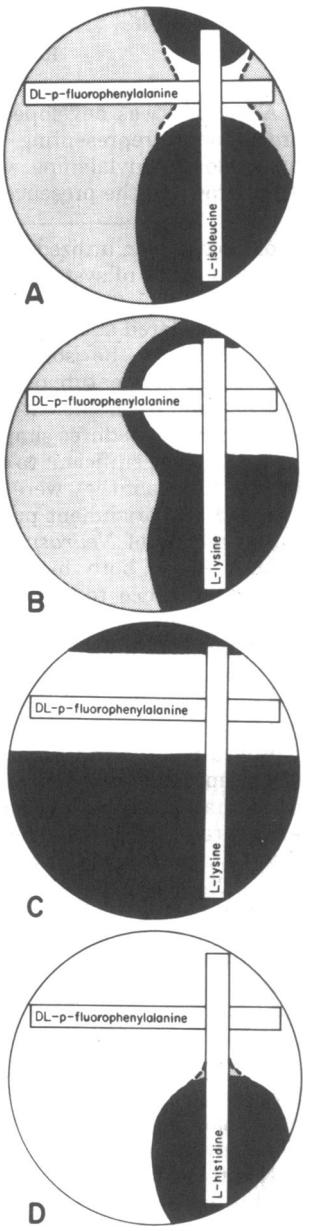

FIG. 2. *Double-gradient plates of: (A) isoleucine bradytroph (UK-71-26); (B) lysine bradytroph (UK-71-7); (C) wild type (74A); and (D) histidine-1 auxotroph (K-83). The clear area represents no growth, the light stipple represents slow leaky growth, and the dark stipple represents heavy growth equivalent to that of the wild type in the absence of FPA.*

Figure 2A represents the growth pattern of an isoleucine-stimulated mutant (UK-71-26). The pattern is typical also of other isoleucine-, leucine-, and some serine-stimulated mutants. There was heavy growth (equivalent to that of the wild type growing on VSGG), near the amino acid strip, which ended in a cruciform clear area at the intersection of the amino acid strip and the FPA strip. The shape of the clear area was presumably due to effective amino acid competition with FPA for uptake. Thus, even very near the FPA strip, the high concentration of amino acid (isoleucine, in this case) protected the cells from FPA inhibition. Away from the amino acid strip there was uniform, slow leaky growth which was not affected by the FPA strip. Obviously when the mutant was furnished with sufficient amino acid for normal growth, it was sensitive to FPA (as shown by the clear zone at the intersection of the two strips); on the other hand, when it was growing in a leaky fashion, it was resistant to FPA, showing no diminution of growth up to and through the FPA strip.

Figure 2B represents the growth of a lysine-stimulated mutant (UK-71-7). The pattern, however, was typical of all of the lysine- and adenine-stimulated mutants. It was also similar to the pattern for tyrosine- and methionine-stimulated mutants. In each case, there was a band of heavy growth, along the amino acid strip, that ended in a clear zone near the intersection of the lysine and FPA strips. At FPA concentrations that were just below the inhibitory concentration, there was a stimulation of heavy growth further out from the lysine strip than usual. This heavy growth curved toward the FPA strip and continued as a very narrow band of growth through the FPA strip. The tyrosine- and methionine-stimulated mutants showed a cruciform clear area similar to that seen with the isoleucine and leucine mutants. This was expected as both trypsine and methionine are effective competitors for FPA uptake, whereas lysine and adenine are not. Away from the amino acid strip, this group of mutants also showed slow leaky growth which was uninhibited by FPA.

Figure 2C shows the pattern of growth of the wild type (74A), and Fig. 2D shows the pattern of *his-1* (K-83). Both showed the expected patterns. The wild type was uniformly inhibited by FPA regardless of the lysine present on the amino acid strip. The histidine mutant (typical of most auxotrophs tested) grew only near the amino acid strip and was inhibited by FPA.

A second set of experiments involved plates containing bradytroph conidia in VSGG agar, with and without the amino acid supplement that stimulated their growth, and with FPA disks added after the agar had set. In each case, no zone of inhibition was formed when the cells grew without supplement, but on the plates with supplement a zone of inhibition similar to that seen with the wild type was observed.

From these results it is obvious that the majority of the bradytrophs isolated by this procedure were resistant to FPA when growing without supplement but were sensitive when supplement was added. Two of the serine-stimulated mutants, however, were not significantly resistant to FPA by these tests. Interesting interactions between the concentration of FPA and amino acid supplement were shown for some of the mutants (lysine, adenine, tyrosine, and methionine stimulated). At FPA concentrations just below the inhibitory concentration, the amino acid requirement was partially, but not completely, relieved. In the same strains there appeared to be a critical concentration of amino acid at which normal growth was obtained but at which the cells remained resistant to FPA—as though they were growing in a leaky fashion (this is indicated by the narrow band of heavy growth which curved up through the FPA strip).

Why should bradytrophs be resistant to FPA when growing in a leaky fashion? The most likely explanation is that the partial deprivation of one amino acid results in increases of the internal pools of other amino acids. This has been shown to be the case in the tyrosine bradytroph acc^{Phe} (3; R. Mason and A. G. DeBusk, personal communication) as well as in amino acid auxotrophs that are being starved of their required supplement (Mason and DeBusk, personal communication). It is also known that the build-up of internal amino acids pools results in *trans*-inhibition of both the neutral and basic amino acid permeases of *Neurospora* (8). In addition, the accumulation of large pools of amino acids could result in their release into the medium, affording a second level of protection by competing with the FPA for any permease activity that might develop.

None of the bradytrophs discussed above has been further characterized with the exception of those stimulated by isoleucine. All of the isoleucine mutants were found to be defective in threonine dehydratase, and the lesion in each case was mapped to linkage group VII.

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