*SYNERGISM OF TWO CYTOPLASMICALLY INHERITED MUTANTS IN NEUROSPORA CRASSA**

By Thad H. Pittenger

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE

Communicated by G. W. Beadle, August 1, 1956

The complementarity of nonallelic nuclear genes in *Neurospora* heterokaryons has been recognized since the early work of Dodge[1] and Beadle and Coonradt,[2] but little is known of interactions between cytoplasmic mutants. The mutations of the particle-bound respiratory system in *Neurospora* offer an opportunity for such a study. The first of these maternally inherited cytoplasmic mutants, mi-1 (*poky*) and mi-3, were reported by Mitchell and Mitchell[3] and Mitchell *et al.*[4] and are characterized by subnormal growth rates and defective cytochrome systems. Biochemical and genetic tests have shown that the two mutants are not identical;[4–8] hence it seemed possible that a cytoplasmic mixture of *poky* and mi-3, a heterocytosome, might restore the normal phenotype. No improvement of the growth rate was observed in mixed cultures, however.[4]

More recently in this laboratory another cytoplasmically inherited mutant, mi-4, has been found that phenotypically resembles *poky*. Both mi-4 and *poky* are transmissible to other strains by heterokaryosis; hence their genetic basis is not merely the absence of some normal cytoplasmic entity but rather the presence of an abnormal one. Although a detailed biochemical analysis of mi-4 has not yet been completed, a difference between mi-4 and *poky* is apparent because they interact in a heterocytosome to produce a phenotype different from either strain alone. The experiments reported here show that an essentially normal growth rate results from cytoplasmic fusion of these two slowly growing strains and that the stimulation cannot be attributed to nuclear interaction.

Description of the Strains.—The spectroscopic appearance of mycelial pads of mi-4 is indistinguishable from that of *poky*[4] when a small grating spectroscope is used; possibly a more refined technique would reveal differences. The rate of oxidation of reduced cytochrome c in the presence of mycelial homogenates, measured with a Beckman spectrophotometer, is similar for mi-4 and *poky* and markedly retarded in comparison to normal strains.

When mi-4 is the conidial parent in crosses, its phenotype is not transmitted, although associated nuclear markers segregate normally. In the reciprocal cross, fertile perithecia have never been produced; thus the maternal inheritance of mi-4 is an extrapolation from its other genetic properties. Fertile crosses have been obtained, however, when the heterocytosome of mi-4 + *poky* was the protoperithecial parent in crosses with either *poky* or wild type. When *poky* was the male parent, all the progeny (*ca.* 10,000 ascospores examined) were phenotypically mutant, but when a wild type was the male parent, up to 1 per cent of the progeny were phenotypically wild; however, this was in the range encountered in similar experiments of *poky* × wild crosses previously reported.[3] Mi-4 arose spontaneously in a lysine-requiring strain (4545)[9] and has been obtained in other genetic backgrounds only by heterokaryosis. Heterokaryons between *lys*, mi-4 and stocks with wild-type cytoplasm have shown the mi-4 phenotype after a variable

period of normal growth. From such heterokaryons, homokaryotic isolates have been obtained free of *lys* nuclei but having the mi-4 cytosome.

The *lys*, mi-4 strain in horizontal growth tubes shows a prolonged lag, then a period of growth at subnormal rates, and eventual cessation of growth. If growth resumes, it again ceases after a variable interval. The cycle of stopping and starting may be repeated several times. Similar cycles are observed with *poky* strains, but the reason for the cyclic behavior remains unknown.

In addition to the rate and duration of uninterrupted growth in horizontal tubes, the growth of the conidia on sorbose medium, used to induce colonial-type growth, has been one of the most useful criteria for recognizing the mi-4 and *poky* phenotypes. When mi-4 conidia are plated on sorbose medium, the majority do not grow, but some produce microcolonies that, except for their mycelial morphology, are reminiscent of the petite colonies of yeast.[10] A high proportion of *poky* conidia are viable, but these, too, produce microcolonies. The mi-4 microcolonies stop growing when small and are seldom viable thereafter, even when transferred to regular medium. The *poky* microcolonies continue to grow slowly, may eventually reach normal size, and are usually viable on transfer. It is not always possible to distinguish *poky* and mi-4 phenotypes from each other, since the degree of expression is influenced by the nuclear genome, but both can always be clearly distinguished from wild type.

For use in the present experiments a *poky* albino, pantothenate-requiring strain (mi-1, 4637, 5531, A)[4, 9] that is heterokaryon positive with *lys*, mi-4, A was selected from a cross of 3627-2a(*poky*)[8] × *pan*, *al*-1, A. This strain, designated "*pan*, *poky*" for this discussion, shows the behavior just described for *poky*. From the *lys*, mi-4 and *pan*, *poky* strains, two other strains were derived, having the *lys* and *pan* (nuclear) genetic background but having normal cytoplasm. The method of obtaining these strains was suggested by the observation that the conidia from newly formed heterokaryons between mi-4 or *poky* and normal strains, taken from a point close to the mixed inoculum, would yield some normal colonies on sorbose agar. Accordingly, controlled inocula were prepared (Pittenger *et al.*)[11] with *lys*, mi-4 or *pan*, *poky* as the minor component and a normal strain with different nuclear markers as the major component, to provide a large admixture of normal cytoplasm. On plating conidia from the resultant heterokaryon, a few normal colonies were obtained that were homokaryotic for *lys* and *pan*. The wild-type condition of the cytoplasm of these isolates was confirmed by growth studies, conidial plating, and spectroscopic examination.

The evidence for synergism of mi-4 and *poky* is the behavior of a number of combinations of the three cytosomes, normal, mi-4, and *poky* and two nuclear types, *lys* and *pan*. The characteristics of these combinations are summarized in Table 1, and more detailed descriptions are given in the sections that follow.

The lys, mi-4 + pan, poky Combination.—This combination was prepared from mixed inoculum[11] and transferred to horizontal growth tubes containing minimal agar supplemented with optimal lysine and calcium pantothenate. The growth rates of the combination and the original strains were measured by the method of Ryan *et al.*[12] at 30° C. As shown in Figure 1, the heterokaryon (and heterocytosome) grows much more rapidly than the component strains. After completion of growth, conidia from the ends of the growth tubes were plated on minimal and

supplemented sorbose medium. A majority of the colonies from the heterocytosome were of normal size on all media within a week after plating, whereas conidia of the two original strains formed only microcolonies in the same period of time. Growth of the heterokaryon and the two homokaryons was compared by growing

TABLE 1

SUMMARY OF THE BEHAVIOR OF A NUMBER OF COMBINATIONS OF THREE CYTOSOMES, NORMAL, mi-4, AND *poky*, AND TWO NUCLEAR TYPES, *pan* AND *lys*

Genotype		Phenotype*	Stability of Phenotype
Nucleus	Cytosome		
pan	*poky*	Mutant	Stable
lys	*poky*	Mutant	Stable
pan + *lys*	*poky*	Mutant	Stable
pan	mi-4	Mutant	Stable
lys	mi-4	Mutant	Stable
pan + *lys*	mi-4	Mutant	Stable
pan	mi-4 + *poky*	Wild	Becomes mutant
lys	mi-4 + *poky*	Wild	Becomes mutant
pan + *lys*	mi-4 + *poky*	Wild	Becomes mutant
pan	Normal	Wild	Stable
lys	Normal	Wild	Stable
pan + *lys*	Normal	Wild	Stable
pan + *lys*	Normal + mi-4	Wild	Becomes mutant
pan + *lys*	Normal + *poky*	Wild	Becomes mutant

* Phenotype refers to initial growth rate and ability of conidia to form normal colonies on sorbose medium but not to spectroscopic analysis.

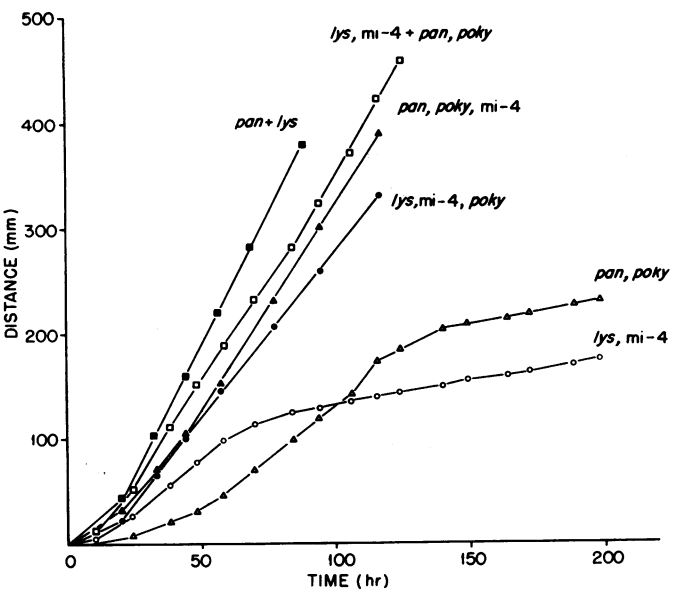

FIG. 1.—Characteristic growth curves on supplemented medium of identical nuclear types, *pan* and *lys*, with various combinations of normal, mi-4, and *poky* cytosomes.

the cultures in supplemented liquid medium for 3 days at 30° C. Comparison of the dry weights of the mycelial pads showed that the heterokaryons had grown more than $2^1/_2$ times as much as the average of the two homokaryons. Despite the striking stimulation of growth, the spectroscopic appearance of mycelial pads of

the heterocytosome was always mutant. Although the heterokaryons may have normal growth rates over distances of 1,000 mm. or more, the normal growth is not permanent, and eventually the stopping and starting phenomenon characteristic of mi-4 or *poky* was also observed in the heterokaryons.

Combinations in Which Both of the Strains Have Normal Cytoplasm.—Dodge[1] and Dodge *et al.*[13] have demonstrated with *N. tetrasperma* that the heterokaryotic vigor observed when two slowly growing strains were combined can be caused by the interaction of genes located in different haploid nuclei of the heterokaryon. Consequently, where a synergistic effect is noted and when the cytoplasm is suspected of having an effect, it must first be shown that nuclear interaction is not the primary cause of the observed results. That nuclear interaction alone could not account for the results in the *lys*, mi-4 + *pan*, *poky* heterokaryon (and heterocytosome) was indicated by the results of similar experiments in which strains of the same nuclear constitution were used, but with the mi-4 and *poky* cytoplasm replaced by normal cytoplasm. In marked contrast to the results just presented, the growth rate of the heterokaryon (Fig. 1) with the normal cytoplasm was no greater than that of the faster-growing component.

Combinations in Which One of the Strains Has Normal Cytoplasm.—Heterokaryons in which one of the component strains has normal cytoplasm, e.g., *lys* + *pan*, *poky* or *lys*, mi-4 + *pan*, initially show normal growth, and the conidia produce normal colonies, but such cultures eventually show the mutant phenotype as expressed by the formation of microcolonies and abnormal growth behavior. The production of conidia that showed the mutant phenotype on plating considerably preceded the retardation in growth.

Lys, mi-4, poky and pan, mi-4, poky Combinations.—Although these experiments indicate that the increased growth rate of the heterokaryons with the heterocytosome was not the result of any nuclear interaction, the most conclusive evidence is the fact that from *lys*, mi-4 + *pan*, *poky* cultures it is possible to isolate from conidia homokaryotic cultures of *lys* and *pan* whose growth rates were nearly wild type (Fig. 1). Where only one nuclear type is present, the increased growth rate must be attributed to the heterocytosome. It should be pointed out that when either homokaryotic or heterokaryotic conidia are isolated from such heterokaryotic cultures and subcultured, the resultant strains may have different growth rates, ranging from essentially normal to those comparable to the original mutant strains. One possible explanation is that the distribution of the two mutant constituents in the conidia results in a range of proportions only some of which are capable of giving normal growth. All such isolates are, however, unstable and revert to the mutant phenotype after prolonged propagation.

Heterokaryotic poky Combinations.—The growth rates of various *poky* heterokaryons as compared to mi-4 + *poky* heterokaryons were those to be expected on the basis of the hypothesis that the observed synergism is primarily the result of the heterocytosome. In Table 2 are given the fastest growth rates achieved on supplemented medium by five homokaryotic strains (all heterokaryon positive) derived from different isolations in addition to several heterokaryons between different *poky* strains and between mi-4 and *poky* strains. None of the growth rates listed for the *poky* heterokaryons persisted for more than 36–48 hours, after which the growth rates were much slower. However, the growth rates of the mi-4 + *poky* hetero-

karyons were constant throughout the 350-mm.-long growth tubes. Although some of the *poky* heterokaryons grew slightly faster than either of the component strains alone, this heterokaryotic vigor was not nearly so great as the synergism shown by the mi-4 + *poky* cultures.

TABLE 2

FASTEST GROWTH RATE ACHIEVED ON SUPPLEMENTED MEDIUM AT 30° C. BY DIFFERENT CYTOPLASMIC AND NUCLEAR COMBINATIONS

Strain	Growth Rate (Mm/Hr)	Strain	Growth Rate (Mm/Hr)
pan, poky No. 14	2.2	*pan, poky* No. 14 + *pan, poky* No. 17	2.4
pan, poky No. 16	1.7	*pan, poky* No. 16 + *pan, poky* No. 17	1.9
pan, poky No. 17	2.2	*pan, poky* No. 17 + *lys, poky* No. 8	2.8
lys, poky No. 8	1.6	*pan, poky* No. 17 + *lys,* mi-4	4.5
lys, mi-4	2.3	*lys,* mi-4 + *lys, poky* No. 8	5.0
pan, poky No. 14 + *pan, poky* No. 16	2.9		

Discussion.—From the evidence presented here, there can be little doubt that the observed synergistic effect on growth is caused by the coexistence of mi-4 and *poky* cytoplasm. The results suggest that in the cytoplasm of wild-type *Neurospora* some cytoplasmic constituent has separate genetic continuity and that *poky* and mi-4 represent different alterations or mutations of this constituent. No critical evidence is at hand to identify this constituent with the particle bearing the terminal oxidase system. Neither mi-4 nor *poky* is the result of an interaction between the nucleus and the normal cytoplasm, since both nuclear types isolated from mi-4 and *poky* cultures will maintain a normal cytoplasmic condition indefinitely. The cytoplasmic elements characteristic of mi-4 and *poky* appear to have their own genetic continuity and are capable, under the conditions of the experiments, of replacing their wild-type analogue. A competitive replacement of wild type by mi-4 or *poky* may account for the instability of the heterocytosome in which either mutant cytoplasm initially coexists with the wild type. A similar competition between mi-4 and *poky* may account for the instability of the heterocytosome and for the failure to find evidence for the transmission of the heterocytosome by crossing.

The synergistic action of the mutants could be explained in one of two ways: either mi-4 and *poky* have complementary action, or they can co-operate to form the normal cytoplasmic constituent. No evidence for normal cytoplasm in the heterocytosome has yet been found, since no stable normal strains have appeared among conidial isolates, even under the same conditions that allow recovery of stable normal strains from cultures with mixed mutant and wild-type cytoplasm. Similarly, when the stimulated heterocytosome is the protoperithecial parent in crosses with *poky*, no wild-type ascospores are recovered. The spectroscopic appearance of the stimulated heterocytosome is mutant, whereas a wild-type appearance would be expected if normal particles were present in large numbers. The interaction of different particles that remain separate is not without precedent, since Tissieres[14] has shown that particles from wild-type *Neurospora*, altered by treatment at alkaline pH, can reconstruct succinate oxidation in vitro when mixed with *poky* particles. An explanation for the synergistic interaction of mi-4 and *poky* must await a detailed comparison of the respiratory systems of the various cytosomes.

The author is indebted to Mrs. Mary Mitchell, who was kind enough to furnish him cultures of *poky*, and to Dr. K. C. Atwood for suggestions concerning the manuscript.

* Work performed under Contract No. W-7405-eng-26 for the United States Atomic Energy Commission.

[1] B. O. Dodge, *Bull. Torrey Botan. Club*, **69**, 75, 1942.
[2] G. W. Beadle and V. L. Coonradt, *Genetics*, **29**, 291, 1944.
[3] M. B. Mitchell and H. K. Mitchell, these PROCEEDINGS, **38**, 442, 1952.
[4] M. B. Mitchell, H. K. Mitchell, and A. Tissieres, these PROCEEDINGS, **39**, 606, 1953.
[5] A. Tissieres and H. K. Mitchell, *J. Biol. Chem.*, **208**, 241, 1954.
[6] F. A. Haskins, A. Tissieres, H. K. Mitchell, and M. B. Mitchell, *J. Biol. Chem.*, **200**, 819, 1953.
[7] A. Tissieres, H. K. Mitchell, and F. A. Haskins, *J. Biol. Chem.*, **205**, 423, 1953.
[8] M. B. Mitchell and H. K. Mitchell, *J. Gen. Microbiol.*, **14**, 84, 1956.
[9] G. W. Beadle and E. L. Tatum, *Am. J. Botany*, **32**, 678, 1945.
[10] P. Slonimaski and B. Ephrussi, *Ann. Inst. Pasteur*, **77**, 47, 1949.
[11] T. H. Pittenger, A. W. Kimball, and K. C. Atwood, *Am. J. Botany*, **42**, 954, 1955.
[12] F. J. Ryan, G. W. Beadle, and E. L. Tatum, *Am. J. Botany*, **30**, 784, 1943.
[13] B. O. Dodge, M. B. Schmitt, and A. Appel, *Proc. Am. Phil. Soc.*, **89**, 575, 1945.
[14] A. Tissieres, *Biochem. J.* (London), **58**, 142, 1954.

---

*MUTATION RATES OF SEVERAL GENE LOCI IN NEUROSPORA**

BY VAL W. WOODWARD

BROOKHAVEN NATIONAL LABORATORY, UPTON, NEW YORK, AND KANSAS STATE COLLEGE, MANHATTAN

Communicated by G. W. Beadle, August 21, 1956

The mutation-rate studies of Stadler [1-3] led him to conclude that genes, with relatively few exceptions, are highly stable. For the *R*, *I*, and *Pr* loci in corn, he reported spontaneous mutation rates of 4.92, 1.06, and 0.11 per 10^4 female gametes, respectively, while the rates for other loci (*Su*, *C*, *Y*, and *Sh*) varied slightly from 0.01 per 10^4 female gametes. Of 1.5×10^6 gametes, he observed no spontaneous mutations of the *Wx* locus.[4] The *R* locus was regarded as "Mutable," and the more stable loci were taken to represent rather normal gene stability.

Singleton[5] recently reported quite different mutation rates for many of the same loci in corn. He reported spontaneous mutation rates of 5.8, 4.4, 5.8, and 1.4 per 10^4 male gametes for the *Pr*, *Sh*, *R*, and *Su* loci, respectively. Singleton attributed the discrepancy between his and Stadler's data to the fact that gene stability depends upon the metabolic state of the plant and also to the hypothesis that there is less selection against certain types of mutation in the male gemetophyte.

Mutation rates have been studied in many organisms. Timofeeff-Ressovsky, quoted by Dobzhansky,[6] reported spontaneous mutation rates that varied from zero and near zero to 5.15 mutations per 10^4 chromosomes for eye-color alleles in *Drosophila*. The spontaneous mutation rates for several genes of man were estimated to vary from 0.1 to 0.8 mutations per 10^4 gametes.[7] In bacteria the spontaneous reverse mutation rates varied from 0.07 to 5.61 mutations per