A GENETIC ANALYSIS OF COLOR MUTANTS OF ASPERGILLUS FUMIGATUS[1]

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THE parasexual cycle in fungi was first discovered in *Aspergillus nidulans* (PONTECORVO 1953) and has since been reported in other species of this genus (ISHITANI, IKEDA and SAKAGUCHI 1956; PONTECORVO, ROPER and FORBES 1953), in *Penicillium chrysogenum* (PONTECORVO and SERMONTI 1954), in *Fusarium oxysporum* forma *pisi* (BUXTON 1956; TUVESON and GARBER 1959), and in *Cephalosporium mycophilum* (TUVESON and COY 1961). Heterocaryosis and the isolation of a prototrophic, diploid heterozygous spore from a heterocaryon involving nutritionally deficient strains constitute the first and second steps, respectively, in demonstrating the parasexual cycle. Mitotic crossing-over or haploidization, or both, may occur during the vegetative multiplication of diploid nuclei so that spores may be obtained which have either diploid nuclei homozygous for certain genetic markers or haploid nuclei containing new combinations of genetic markers. Consequently, methods are now available for genetic studies using fungi lacking a sexual stage (PONTECORVO 1953).

Numerous color mutants were obtained in *Aspergillus fumigatus* Fres., the usual cause of aspergillosis, a disease characterized by inflammatory granulomatous lesions in the skin, external ear, nasal sinuses, bronchi, or lungs. Since this species lacks a sexual stage, a genetic study of the color mutants required the methods developed in demonstrating the parasexual cycle.

MATERIALS AND METHODS

A culture of strain 188 of *A. fumigatus* was obtained from DR. W. R. MARTIN, Department of Microbiology, University of Chicago. The pathogenicity of this strain was not determined.

Cultures exhibited excellent growth and produced numerous dark green uninucleate spores (YUILL 1950) on either a complex or defined medium. The complex medium contained 0.5 percent yeast extract, 2.0 percent glucose, and 1.5 percent agar (Difco). The defined medium (BRAUN 1950) had the following composition: KNO_3, 1.5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 2.4 g; KH_2PO_4, 2.4 g; $FeCl_3$, 40 mg; $MnSO_4$, 4 mg; H_3BO_4, 4 mg; $CuSO_4$, 0.4 mg; $ZnSO_4$, 0.4 mg; and glucose, 20 g in one L distilled water. Cultures were incubated at 33° to 36°C.

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Conidia were harvested by one of two techniques. Plates of sporulating cultures were flooded with an aqueous solution (0.01 percent) of Tween 80, the surface of the culture was stroked with a sterile loop, and the spores were collected with a sterile pipette. The alternate method involved the transfer of large pieces of mycelium to a test tube containing a solution of Tween 80 which was then vigorously agitated. To obtain single spores, the spore suspension was sucked into a Pasteur pipette and then forcefully ejected not less than 30 times. The number of spores was estimated, using a hemocytometer. Approximate dilutions of the suspension were spread on complex medium to determine the number of spores yielding colonies. In certain experiments, the spore suspensions were filtered through sterile cotton to eliminate hyphal fragments prior to estimating the number of spores.

From 1.2×10^5 to 6.0×10^6 spores in two ml water were irradiated with ultraviolet light for four to eight min in a light-proof box with six 8-watt G.E. "sterilamps" mounted 80 cm above the target area. The dosages used were lethal for 99.0 to 99.9 percent of the spores. Exposed spores were kept in darkness for two hr to preclude photoreactivation. Appropriate dilutions of the suspension were added to complex medium to obtain isolated colonies which were then transferred to make a grid of 26 colonies. Spores from these colonies were transferred to plates of defined medium supplemented with amino acids, purines, pyrimidines, vitamins, or growth factors, using a multineedle inoculating device; procedures for determining specific nutritional requirements have already been described (PONTECORVO 1953).

Two methods were used to obtain heterocaryons. In the first or frontier method, small sections of mycelium at the boundary between two mutant colonies growing on complex medium were transferred to defined medium and outgrowths were again transferred to this medium. In the second or mat method, spores of two nutritionally deficient strains were dusted on the surface of sterile distilled water in a test tube containing complex medium. The resulting mycelial mat was shredded and plates of defined medium were seeded with the fragments. Outgrowths from fragments were transferred to defined medium.

RESULTS

Color mutants: Irradiated spores of the parental strain were plated on complex medium and color mutants were obtained by inspection. The frequency of such mutants was not determined. Except for mutant 10 which was dilute green and required leucine, the color mutants were not nutritionally deficient. Irradiated spores from the dilute green mutant yielded two color mutants: 10a, an albino colony requiring lysine and leucine; and 10c, a turquoise colony requiring both isoleucine and valine as well as leucine. Spores of a prototrophic buff strain were irradiated and yielded a prototrophic mustard mutant. Nutritionally deficient mutants obtained from both the buff and mustard strains retained their respective color. Mutants which did not change color when nutritional deficiencies were added have been termed prototrophic-color mutants; color mutants which were nutritionally deficient when initially isolated have been termed auxotrophic-

color mutants. Strains 10, 10a, and 10c were the only auxotrophic-color mutants which were detected. A list of the mutant strains used in this investigation appears in Table 1.

Spore color at frontiers: Two groups of color mutants were recognized on the basis of spore color at frontiers between colonies. Group I included the taupe, cinnamon, brown, white, and albino strains and Group II, the buff, mustard, blue, chartreuse, and creamy strains. With certain exceptions, green spores were observed at the frontier between a strain from each group or between strains in Group I; green spores were not seen at the frontier between strains in Group II. The frontier between taupe (Group I) and mustard (Group II), between taupe and brown (Group I), or between white (Group I) and albino (Group I) did not yield green spores. Forced heterocaryons involving nutritionally deficient mutants are required to demonstrate that these combinations will not yield green spores. The dilute green *leu* or turquoise *leu ilv* strains were not used since these colors were difficult to distinguish from green at frontiers.

Diploid strains: Spores were harvested from four heterocaryons involving strains with different colors and nutritional deficiencies and plated on defined medium. Colonies which did not sector on this medium were derived from diploid spores. The frequency of diploid colonies was not determined. Diploid colonies from heterocaryons involving the albino and buff or mustard strains or the turquoise and mustard strains had green spores; diploid colonies from the heterocaryon involving the yellow and buff strains had yellow-buff spores.

Recombinants from diploid strains: Two methods were used to obtain spores with recombinant nuclei; both methods involved a search for spores with a color other than that of the diploid strain. In the first method, diploid colonies were scanned, using a dissecting microscope, and spores with a recombinant color were

TABLE 1

Mutant strains of Aspergillus fumigatus

Strain	Origin	Color on complex medium	Nutritional requirements*
188	wild type	dark green	none
10	188	dilute green	*leu*
10a	10	albino	*lys leu*
10c	10	turquoise	*ilv leu*
11	188	buff	none
11a	11	buff	*ade*
11d	11	mustard	none
11d–11	11d	mustard	*pan*
20	188	blue	none
21	188	chartreuse	none
22	188	creamy	none
23	188	taupe	none
24	188	cinnamon	none
25	188	brown	none
26	188	white	none

* leucine–*leu*, lysine–*lys*, isoleucine and valine–*ilv*, adenine–*ade*, pantothenic acid–*pan*.

seeded on complex medium to obtain colonies. In the second method, appropriate dilutions of a suspension of spores from diploid colony were plated on complex medium to obtain isolated colonies whose color was different from the diploid or the original dark green strain. A few colonies appeared to differ slightly from the dark green strain and these were designated as "green" colonies. The nutritional requirements of these colonies may have been responsible for the slight difference in color. Such recombinants could not be detected by the first method. The frequency of spontaneous recombinant spores was relatively low.

Diploid spores were also plated on complex medium containing 0.01 percent DL-para-fluoro-phenylalanine (FPA) which had been reported to increase the number of haploid sectors in diploid colonies of *A. niger* (LHOAS 1961). Outgrowths from diploid colonies of *A. fumigatus* were usually recombinants. The color and nutritional requirements of recombinant spores from four diploid strains of *A. fumigatus* are presented in Table 2.

All lysine-requiring recombinants from two diploids involving the albino *lys leu* strain were albino. Except for the albino *lys* recombinants from Diploid 1 and for one albino *lys leu* recombinant from Diploid 2, albino recombinants yielded green spores at frontiers. The albino *lys* recombinants gave buff spores and the albino *lys leu* recombinant, mustard spores.

Buff recombinants were not obtained from diploids involving the mustard strain. Unexpected color phenotypes were found among the recombinants from two different diploids. Diploid 3 gave yellow recombinants and Diploid 4, light buff recombinants. Since these recombinants did not yield green spores at frontiers, they were classified as prototrophic-color phenotypes. Both yellow and light buff colonies yielded fewer spores than the buff or mustard colonies; the difference could also be detected by examining the colonies, using a dissecting microscope.

DISCUSSION

The origin of the auxotrophic-color mutants, exemplified by the albino-*lys* mutant, may have at least three different explanations: (a) double mutations, (b) one mutation involving a direct precursor of the original dark green color and, coincidentally, leading to a nutritional deficiency, or (c) one mutation responsible for the nutritional deficiency and coincidentally altering the original color. The third explanation assumes that the nutritional deficiency is not directly involved as an intermediate in pigment biosynthesis.

Since all albino recombinants from two diploid strains required lysine, a mutation would have had to occur in two closely linked genes, one for the nutritional deficiency and the other for the altered color. A similar argument would have to apply to the origin of the turquoise strain requiring both isoleucine and valine as well as leucine. Auxotrophic-color mutants grown on defined medium supplemented with the nutritional requirements did not yield green spores. Consequently, it is difficult to assume that the nutritional requirements were directly involved in pigment biosynthesis. The auxotrophic-color mutants may have resulted from a single mutation responsible for a nutritional deficiency and for a

TABLE 2

Recombinants from four diploid strains

Color	Requirements	Number of recombinant spores		
		Spontaneous	FPA*	Total
	Diploid 1: albino *lys leu*/buff *ade*			
"green"	*ade*	2	1	3
dil. green	*leu*	1	1	2
buff	none	128	23	151
buff	*ade*	6	4	10
buff	*leu*	2	0	2
albino	*lys*	2	0	2
albino	*lys leu*	6	28	34
albino	*lys leu ade*	1	3	4
	Diploid 2: albino *lys leu*/mustard *pan*			
mustard	none		37	37
mustard	*pan*		16	16
albino	*lys leu*		74	74
albino	*lys leu pan*		72	72
	Diploid 3: turquoise *ilv leu*/mustard *pan*			
dil. green	*leu*	9	27	36
dil. green	*leu pan*	2	17	19
"green"	*ilv*	1	0	1
"green"	*pan*	2	1	3
"green"	*ilv pan*	0	1	1
turquoise	*ilv leu*	1	0	1
turquoise	*ilv leu pan*	2	3	5
mustard	none	74	45	119
mustard	*pan*	9	18	27
mustard	*pan ilv*	1	0	1
yellow	none	5	4	9
yellow	*ilv*	1	1	2
yellow	*pan*	0	3	3
	Diploid 4: yellow *ilv*/buff *ade*			
buff	*ade*		56	56
buff	*ilv*		2	2
lt. buff	none		12	12
lt. buff	*ade*		26	26
lt. buff	*ade ilv*		1	1
mustard	none		20	20
mustard	*ade*		25	25
mustard	*ilv*		1	1
yellow	none		9	9
yellow	*ade*		18	18

* DL-para-fluoro-phenylalanine.

coincident alteration of color as a consequence of a secondary block in pigment biosynthesis. An inhibition or alteration of pigment biosynthesis might result from an accumulation of one or more metabolites above the normal level.

Two types of color mutants in *A. fumigatus* were recognized on the basis of

spore color at frontiers. The color of uninucleate spores of a heterocaryon may be determined *solely* by the genotype of each spore or by the genotypes included in the heterocaryon. PONTECORVO (1946) has termed the former case "autonomous gene action" and the latter, "nonautonomous gene action." The color mutants of *A. fumigatus* assigned to Group I would be classified as nonautonomous and those in Group II as autonomous.

A nonautonomous color mutant may yield spores with a different color when grown on medium supplemented with culture filtrates from other mutant strains (ISHITANI and SAKAGUCHI 1956) or in heterocaryons involving other mutant strains. In the former situation, the source of diffusible material which alters the color of the nonautonomous mutant would be exogenous and in the latter, endogenous. The auxotrophic-color mutants, represented by the albino-*lys* strain, and certain prototrophic-color strains in *A. fumigatus* were nonautonomous. The green spores at the frontier between these prototrophic-color mutants constituted a phenotypic alteration in spores from each strain by the mutual repair of the lesions in pigment biosynthesis, comparable to mutual syntrophism for nutritionally deficient mutants. In heterocaryons involving an auxotrophic-color mutant, however, the green spores presumably resulted from the utilization of the accumulated metabolite or metabolites which were responsible for the secondary block in pigment biosynthesis. Consequently, two different mechanisms may be responsible for nonautonomous gene action in color mutants of *A. fumigatus*.

All except three albino recombinants from diploid strains involving the albino strain displayed green spores at frontiers. Two recombinants gave buff spores and one, mustard spores. These observations suggest that albino is epistatic to buff and mustard. With the relief of the secondary block in the heterocaryon at the frontier, pigment biosynthesis proceeded either to the buff or to the mustard color. If buff or mustard were absent, pigment biosynthesis in the albino spores would have proceeded to the dark green color. It should be noted that both buff and mustard are autonomous color mutants.

Although green spores were found at the frontier between different nonautonomous color strains, it was difficult to establish that both strains were contributing to the frontier. By using a nonautonomous prototrophic color strain and an albino, buff recombinant, the frontier yielded spores from each strain as well as green spores from the prototrophic strain and buff spores from the recombinant.

The origin of the mustard, yellow, and light phenotypes merits discussion. All three phenotypes are autonomous. Mustard had been obtained by irradiating buff spores. The diploid strain involving mustard and turquoise gave yellow but not buff recombinants. Finally, the diploid strain involving buff and yellow gave buff, light buff, yellow and mustard but not green recombinants. Two assumptions provide the basis for a reasonable explanation of these observations: (a) mustard is an allele of buff and (b) a spontaneous mutation for reduced sporulation (*rs*) as well as dilution of color occurred in the diploid strain involving mustard and turquoise. The following genotypes are proposed for the re-

combinants from the diploid strain with buff and yellow: buff–*bu*+, light buff–*bu rs*, mustard–*bu*^{mu}+, and yellow–*bu*^{mu}*rs*. The diploid strain would have the following genotype with respect to color: *bu*^{mu}*rs*/*bu*+.

Although linkage studies were not the primary objective of this investigation, the recombinants from two diploids provided evidence indicating a linkage of certain genes. The albino-lysine, leucine, and buff loci constituted one linkage group. Approximately two percent of the albino recombinants carried either the buff or mustard alleles as a consequence of somatic crossing-over.

The frequency of recombinants requiring either adenine or both isoleucine and valine from diploids which were heterozygous for one of these loci warrants comment. Approximately 30 percent of the nutritionally deficient recombinants from Diploid 1 and approximately 98 percent from Diploid 4 required adenine; approximately 11 percent of the nutritionally deficient recombinants from Diploid 3 and approximately two percent from Diploid 4 required both isoleucine and valine. Furthermore, prototrophic recombinants from Diploid 1 constituted approximately 72 percent of the recombinant population. Genetic markers which are subject to strong selective forces when present in haploid nuclei in the mycelium of diploid strains should not be used for linkage studies in *A. fumigatus*. Since recombinants may result from somatic crossing-over or haploidization, future genetic studies in this species will require strains with marker genes linked with the color mutants.

SUMMARY

Prototrophic-color and auxotrophic-color mutants were obtained by ultraviolet irradiation of uninucleate spores of *Aspergillus fumigatus*, an imperfect fungus. The former presumably represent mutations involving direct precursors of the parental dark green spore-color and the latter, mutations resulting in a nutritional deficiency and, coincidentally, an alteration of the parental color. In the latter mutations, an accumulation of metabolites above normal levels may have inhibited pigment biosynthesis as a result of a secondary block.

One group of color mutants was autonomous and the second group was nonautonomous, yielding green spores at frontiers or in heterocaryons. Two different mechanisms for nonautonomy were proposed. Spore color at frontiers or in heterocaryons between nonautonomous prototrophic-color and auxotrophic-color mutants was explained by assuming diffusion of metabolites to repair the lesion in the former mutants and utilization of accumulated metabolites in the latter mutants.

Three of the four diploid strains which were studied had green spores as in the wild type and the fourth, buff spores. Recombinant spores were obtained from diploid colonies either on complex medium, or, with higher frequency, on this medium supplemented with DL-para-fluoro-phenylalanine.

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