Genet. Res. Camb. (1963), **4**, *pp.* 276–283
Printed in Great Britain

Formal and physiological genetics of ascospore colour in *Aspergillus nidulans*

By D. APIRION

Department of Genetics, The University, Glasgow

(*Received* 21 *December* 1962)

1. INTRODUCTION

A combination of fine genetic analysis with tetrad analysis is desirable for an understanding of recombination at the intragenic level. Tetrads in which intragenic recombination has occurred are very rare however, and 'blind' dissection of huge numbers of tetrads in order to identify these few is not very rewarding. A practical solution to this difficulty is a system in which the rare tetrads can be selected from the mass and the analysis limited to them. Such a method was described by Lissouba & Rizet (1960), Rizet, Lefort, Engelmann, Lissouba & Mousseau (1960) and Lissouba (1961). In heteroallelic crosses between mutants of *Ascobolus immersus* with colourless ascospores rare tetrads containing coloured spores could be identified visually among the many thousands of colourless tetrads.

The present paper reports the isolation of ascospore colour mutants in *Aspergillus nidulans* with the object of applying to this organism, with its more versatile and better explored genetics, the approach used with *Ascobolus immersus*. Unfortunately, the ascospore colour mutants of *Aspergillus nidulans* found so far, and described in the present paper, turned out to be unsuitable for the initial purpose. The genes identified which affect pigment production in the ascospores produce effects which are not cell localized, i.e. they are, in the classical terminology, 'non-autonomous' in action (Sturtevant, 1920; Ephrussi, 1938). The results reported here are, however, interesting in other respects.

Aspergillus nidulans is a homothallic ascomycete multiplying asexually by means of uninucleate haploid conidia and sexually by means of ascospores. The four products of meiosis divide mitotically in the ascus and each of the eight haploid nuclei is included in one of the eight dark red ascospores. There are about 10,000 asci in one fruiting body (perithecium). The perithecium is spherical, about 0·5 mm. in diameter, and has a dark red outer wall.

2. MATERIALS AND METHODS

Techniques and symbols not described here may be found in the papers by Pontecorvo, Roper, Hemmons, Macdonald & Bufton (1953), Pontecorvo & Käfer (1958) and Käfer (1958).

Ascospore colour mutants were obtained by treating conidial suspensions of the strain *y*; *w2*; *s12* with UV or HNO_2 (Siddiqi, 1962). Treated conidia were plated

on complete medium to give 30–50 colonies per dish. The colonies produced mature perithecia after 10–14 days. A few perithecia from each colony were squashed between a slide and a cover slip in a drop of lysol and examined with the naked eye, and with the microscope when necessary.

3. RESULTS

(i) *Mutants*

Eleven mutants were isolated; six differed from the wild-type in having dark blue ascospores, and five in having white (colourless) ascospores. The blue mutants were given the general symbol *bl* and the white mutants the general symbol *cl*. Four *bl* mutants (Nos. 1–4) and five *cl* mutants (Nos. 1 and 3–6) were studied in the work described here. The dark blue colour of *bl* mutants slowly changed to red over a period of a month or more. The *cl* mutants varied from completely colourless in the case of *cl4* to distinctly pink in the case of *cl1*. The wall of the perithecia of *cl* strains was pale pink, so that under a low-power dissecting microscope they were easily distinguishable on the surface of a colony even without picking them up.

(ii) *Formal genetics of the mutants*

bl *mutants* (*blue ascospores*)

In crosses with strains having wild-type ascospores all four of the *bl* mutants showed a 1:1 segregation of blue versus red ascospores among the colonies originating from ascospores of crossed origin (Table 1).

Table 1. *Crosses between blue ascospore mutants* (blx) *and wild-type* (bl^+)

Mutant used in the cross	Number of colonies with blue ascospores	Number of colonies with red ascospores	Total
bl1	46	45	91
bl2	36	43	79
bl3	42	35	77
bl4	38	43	81
Total	162	166	328

Crosses were of the type: $y;w2;s12;blx \times paba1$. In each case ascospores from a single hybrid perithecium were plated on complete medium.

In crosses of *bl1*, *bl3* and *bl4* with *bl2* no colonies with red ascospores, that is wild-type recombinants, were obtained among 200 colonies examined from each cross. Thus these mutants are closely linked or allelic. Tests of *bl* mutants in diploid heterozygotes showed that *bl* mutants are recessive to bl^+ (Table 4). Tests of two of them (*bl1* and *bl2*, Table 4) in diploids showed that these two do not complement in the *trans* arrangement, i.e. they are allelic. The other two-by-two combinations of *bl* mutants have not been tested.

By haploidization of a diploid between *bl1* and tester strain MSD carrying markers in each of the eight chromosomes (Forbes, 1959, and unpublished), *bl1* was located in linkage group II, but crosses involving other markers of this linkage group covering most of it (*ad23*, *Acr1*, *w3*, *ribo6*, *thi4*, *pu*, *ni3*, *ad3*, *lac5*) did not reveal meiotic linkage between *bl1* and any of them. So the *bl* locus adds at least 50 units to this already long linkage group.

cl *mutants* (*colourless ascospores*)

In crosses with a strain having wild-type ascospores, the colourless mutants *cl4*, *cl5* and *cl6* gave a 1 : 1 segregation of red versus white ascospores (Table 2). Crosses

Table 2. *Crosses between colourless ascospore mutants* (clx) *and wild-type* (cl^+)

Mutant used in the cross	Number of colonies with: colourless ascospores	red ascospores	Total
cl4	33	29	62
cl5	45	41	86
cl6	56	60	116
Total	134	130	264

Crosses were of the type: *y*;*w2*;*s12*;*clx* × *bi1*;*meth1*. In each case ascospores from a single hybrid perithecium were plated on complete medium.

of each of the mutants *cl1*, *cl3*, *cl5* with *cl6* gave no wild-type recombinants in about 100 colonies originating from ascospores of crossed origin, while all four gave a 3 : 1 segregation of white to red ascospores in crosses with *cl4* (Table 3). It was concluded that *cl4* identifies one locus and the other four mutants another locus or closely

Table 3. *Recombination between independently arisen colourless ascospore mutants* (cl)

Mutants used in the cross	Number of colonies with: colourless ascospores	red ascospores	Total
cl4 × *cl1*	134	60	194†
cl4 × *cl3*	204	58	262†
cl4 × *cl5*	201	70	271†
cl4 × *cl6*	121	45	166†
cl6 × *cl1*	89	0	89
cl6 × *cl3*	132	0	132
cl6 × *cl5*	117	0	117

† Not significantly different from the results (3 : 1) expected in the case of independent segregation. Thus *cl*4 identifies one locus and the other four identify another unlinked locus.

Crosses were of the type: *bi1*;*clx* × *cly*;*y*;*w2*;*s12*. Ascospores from a number of perithecia from each cross were plated on minimal medium on which only bi^+;s^+ recombinants can grow.

linked loci. Mitotic haploidization from diploids with tester strain MSD located *cl4* in linkage group IV and *cl6* in linkage group I. Two further crosses, *cl6 bi1*; *w2* × *pro1 paba1 y* and *cl6 bi1*; *w2* × *ribo1 an1 ad14 y*; *meth1 pyro4*, located *cl6* on the left arm of linkage group I about 4 units from *ribo1*. Whether *cl6* was proximal or distal to *ribo1* could not be determined from the data obtained from these crosses. *cl4* was tested for linkage with all the known loci in linkage group IV (*meth1*, *fr1*, *pyro4* and *paba22*). The results suggested loose linkage distal to *meth1*.

Dominance and epistasis of mutants

Some of the dominance relationships of ascospore colour mutants with the wild-type alleles and with one another were investigated by synthesizing diploids (Roper, 1952) heterozygous and homozygous for *bl* and *cl* mutants in combination with wild-type and among themselves. The results (Table 4) showed that the *bl* and *cl* mutants tested are recessive to wild-type.

Table 4. *Ascospore colour of diploids heterozygous and homozygous for ascospore colour mutants*

Combination	Type of diploid	Colour of the ascospores of the diploid
†MSD / *paba1*; *w2 bl1*	bl^+ / *bl1*	red
MSD / *paba1*; *w2 bl4*	bl^+ / *bl4*	red
MSD / *cl6 bi1*; *w2*	cl^+ / *cl6*	red
MSD / *bi1*; *w2*; *cl4*	cl^+ / *cl4*	red
bi1; *w2*; *s12*; *cl4* / *paba1 y*; *bl1*; *s12*	*cl4* / *bl4*	red
bi1; *w2*; *s12*; *cl4* / *cl6 an1 ad14 paba1 y*	*cl4* / *cl6*	red
y; *bl1*; *s12* / *paba1*; *w2 bl1*	*bl1* / *bl1*	blue
y; *bl1*; *s12* / *paba1*; *w2 bl2*	*bl1* / *bl2*	blue
cl6 bi1; *w2*; *s12* / *cl6 an1 ad14 paba1 y*	*cl6* / *cl6*	colourless

† MSD is a strain which has markers in each of the eight linkage groups (Forbes, unpublished).

Two crosses between a blue (*bl2*, linkage group II) and two colourless mutants (*cl3* and *cl1*, linkage group I) showed the expected independent segregation. Both colourless mutants were epistatic to the blue mutant, as indicated by a 1:2:1 ratio of colonies with red: colourless: blue ascospores in these crosses (Table 5). Epistasis was confirmed by crossing each of four colourless isolates which also required thiosulphate (*s12*) from the cross *paba1*; *bl2* × *cl3 y*; *w2*; *s12* with strain *paba1*, which has wild-type (red) ascospores. From these crosses $paba^+$; s^+ recombinants were selected by plating suspensions of ascospores on minimal medium. One of these four crosses yielded recombinant colonies with blue ascospores. This indicated that the isolate used in this cross had the double mutant genotype: *cl3*; *bl2*. Among the 78 colonies of this cross, 45 had colourless ascospores, 20 red and 13 blue.

Table 5. *Crosses between blue* (bl) *and colourless* (cl) *ascospore mutants*

Type of cross	Number of colonies with colourless ascospores	blue ascospores	red ascospores	Total
bl2 × *cl3*†	232	166	120	518
bl2 × *cl1*	38	15	19	72

† Many colonies did not form mature ascospores and were therefore classified by the colour of the perithecial wall; such a classification is liable to error.

Crosses were of the type: *paba1*; *bl2* × *clx y*; *w2*; *s12*.

Ascospores from a number of perithecia from each cross were plated on a minimal medium, on which only *paba*$^+$; *s*$^+$ recombinants can grow.

(iii) *Physiological genetics of ascospore colour*

The technique used in analysing crosses between strains differing in ascospore colour is based on the fact that the 10,000 or so asci of an individual perithecium are almost invariably either all of crossed origin or all of selfed origin, a fact which has been utilized in the technique of 'perithecium analysis' (Pontecorvo *et al.*, 1953). The three types of perithecia can be distinguished easily if one parent has the genotype *w*; *y* and the other *w*$^+$; *y*$^+$. White (*w*) is epistatic to *y*$^+$/*y* (yellow conidia). If a small sample of an ascospore suspension prepared from a single perithecium is streaked on complete medium and incubated until conidia colour is developed, it normally gives only one of three patterns, either pure green, pure white, or mixed yellow, white and green. Streaks of the first two types indicate a selfed perithecium and streaks of the third type a hybrid perithecium.

Table 6. *Colour of ascospores of perithecia of selfed and crossed origin in crosses of type* bl × bl$^+$ *and* cl × cl$^+$

Cross	Type of cross	Type of perithecium	Phenotype of ascospores in perithecium: white	red	blue	Total No. of perithecia
y; *w2 bl1*; *s12* × *bi1*; *bl*$^+$; *meth1*	*bl1* × *bl*$^+$	selfed†	0	3	6	9
		crossed	0	5	2	7
cl6 y; *w2*; *s12* × *cl*$^+$ *bi1*; *meth1*	*cl6* × *cl*$^+$	selfed†	51	13	0	64
		crossed	2	22	0	24

† The genotype of the selfed perithecia corresponded in all cases to the genotype of the parent with he same colour of ascospores.

In the present experiments the two parents also differed in genotype with respect to ascospore colour, and in addition to the test described above, a sample of ascospores was examined microscopically to determine the phenotype of the ascospores.

The ascospores analysed in this way were from crosses of types *bl* × *bl*$^+$, *cl* × *cl*$^+$ and *bl* × *cl*. Segregation of wild-type and mutant ascospores was never found,

either in crossed or, understandably, in selfed perithecia. The ascospores of a perithecium were all of the same colour, irrespective of their genotypes. Thus, the genes tested so far determining ascospore colour variation are 'non-autonomous' in action. Moreover, the wall of each perithecium (crossed or selfed) is of the same colour as the walls of the ascospores contained in it, and this colour can be wild-type or that of either parental strain—even in the case of crossed perithecia (Tables 6 and 7).

Table 7. *Colour of ascospores and perithecia of selfed and crossed origin from the cross* cl3 paba1 × y; w2 bl2; s12 (cl3 × bl2)

Phenotype of perithecium and ascospores	Selfed *cl3*	Selfed *bl2*	Crossed	Total†
Colourless	42	0	16	58
Blue	0	17	39	56
Red	10	4	39	53

† About equal numbers of perithecia of each phenotype were tested. The actual proportions in this cross were: red about 10%, colourless about 40%, and blue about 50%.

In detail, the results (Table 7) of crosses between strain *cl3 paba1* (with green conidia and colourless ascospores) and strain *y*; *w2 bl2*; *s12* (with white conidia and blue ascospores) were as follows:

1. All three classes of perithecia—with colourless or red or blue ascospores—occurred. Their proportions varied in replicates of the same cross.

2. Both crossed and selfed perithecia were found among all three classes.

3. Among *selfed red* perithecia, both parental genotypes (*cl3 paba1* and *y*; *w2 bl2*; *s12*) were found, while among *selfed colourless* or *selfed blue* perithecia only one parental genotype, in each case the parental type with the corresponding ascospore genotype. That only parental phenotypes occur in selfed perithecia, and that this phenotype always corresponds to the genotype of the selfed ascospores, was supported by classifying selected red perithecia from a cross of type *cl3* × *cl*$^+$ (*cl3 y*; *w2*; *s12* × *cl*$^+$ *bi1*; *meth1*). This cross gives, of course, white and red perithecia. One hundred and seventy-two red perithecia were picked and analysed (using the technique mentioned above). One hundred and eight of these 172 perithecia were of crossed origin and 64 were of selfed origin. All the 64 of selfed origin were selfed of the parental genotype (*cl*$^+$) producing red perithecia. There were no selfed red perithecia having the *cl* parental genotype.

4. DISCUSSION

Aspergillus nidulans is homothallic and has differentiated male and female organs like heterothallic *Aspergilli* (Adams, unpublished) and the asci originate from dikaryotic ascogenous hyphae (Elliott, 1960). Furthermore, practically all the asci (10,000 or so) in each perithecium are either of crossed or of selfed origin (Pontecorvo *et al.*, 1953). To account for this it was assumed that one 'male' nucleus and one

'female' nucleus enter into conjugated divisions to give rise to all the dikaryotic ascogenous hyphae and therefore to all the asci (Pontecorvo *et al.*, 1953). On the basis of the preceding facts and of what is known from other ascomycetes (e.g. Martens, 1946), let us see how the observations of the present work can be interpreted.

It will be assumed that:

1. The primordium of the female organ—protoperithecium—begins with only a few nuclei.
2. The male organ contributes only *one nucleus*. This nucleus and *one* of the female nuclei enter into conjugated divisions to give rise to the dikaryotic ascogenous hyphae.
3. Side by side with the development of the ascogenous hyphae, the other nuclei —exclusively female—of the protoperithecium multiply and ultimately give rise to the other parts of the perithecium, such as the perithecium wall, which are therefore female.

Thus, while the crossed or selfed origin of the asci in a perithecium is determined by the particular pair of nuclei—one male and one female—which entered into conjugated divisions, the colour of the ripe perithecium wall and of the ripe ascospores (in the particular system of genes investigated here) is determined by the genotypes of the nuclei present in the protoperithecium.

In a cross between blue and colourless strains ($bl \times cl$) in which the hyphae were largely heterokaryotic, the protoperithecial primordium might contain nuclei of either or both types present in the heterokaryon. Thus a ripe perithecium, derived from a heterokaryotic protoperithecium, would usually be red (wild-type), while the asci of this perithecium could be either crossed or selfed, and if selfed, of either parental type depending on the genotype of the nuclei which entered into conjugated divisions. On the other hand, a ripe perithecium, derived from a homokaryotic protoperithecium, would have a parental phenotype (colourless or blue), but the asci of such a perithecium could again be either crossed or selfed depending on the male nucleus, but if selfed they must be of the corresponding genotype, i.e. the same genotype as the nuclei in the protoperithecium.

The same considerations are valid and compatible with the observations in crosses of the type $bl \times bl^+$ and $cl \times cl^+$.

A protoperithecium could, conceivably, be heterokaryotic but with only one nucleus of one parental type and all the others of the other parental type. If this single nucleus were the female contribution to the ascogenous hyphae, and therefore to all the asci, and if in addition the male nucleus contributed to the ascogenous hyphae were of the same parental type, the maternal parts of the perithecium would be left with nuclei all of one parental type while the asci would all be selfed of the other parental type. The fact that perithecia of this type were not found in this investigation can be explained by assuming, either that the number of female nuclei in the protoperithecium is considerable, or that between the formation of the primordium of the protoperithecium and the time when the male nucleus is contributed all nuclei divide once or more.

5. SUMMARY

By nitrous acid or UV treatment ascospore colour mutants of two kinds, blue and colourless, were obtained in *Aspergillus nidulans* (wild-type has red ascospores). Four blue mutants were located in linkage group II within 0·5 unit of one another (*locus symbol*: *bl1*). Of the colourless mutants, four were located in linkage group I within one unit of one another (*locus symbol*: *cl6*), and one in linkage group IV (*locus symbol*: *cl4*). In diploids the mutants were recessive. Colourless was epistatic to blue.

In crosses these characters behaved as 'non-autonomous' both in the ascospores and in the asci; all the ascospores of the asci in one perithecium as well as the perithecium wall were of the same colour. In crosses between strains with blue perithecia and strains with colourless perithecia, red, blue and colourless perithecia were found; each type included both crossed and selfed perithecia. Red selfed perithecia were of either parental genotype but blue or colourless selfed perithecia always had the corresponding genotype.

The phenotype of the perithecium (perithecial wall and ascospores) is considered to be determined by the homo- or heterokaryotic constitution of the protoperithecium which gave origin to it.

I am grateful to Mr E. Forbes for training me in *Aspergillus* genetics and for advice on this problem.

This work was supported by the 'Alexander Milman' Prize from the Hebrew University of Jerusalem.

REFERENCES

Elliott, C. G. (1960). The cytology of *Aspergillus nidulans*. *Genet. Res.* **1**, 462–467.

Ephrussi, B. (1938). Aspects of the physiology of gene action. *Amer. Nat.* **72**, 5–23.

Forbes, E. (1959). Use of mitotic segregation for assigning genes to linkage groups in *Aspergillus nidulans*. *Heredity*, **13**, 67–80.

Käfer, E. (1958). An 8-chromosome map of *Aspergillus nidulans*. *Advanc. Genet.* **9**, 105–145.

Lissouba, P. (1961). Ph.D. Thesis, Paris University.

Lissouba, P. & Rizet, G. (1960). Sur l'existence d'une unité génétique polarisée ne subisant que des échange non réciproques. *C. R. Acad. Sci., Paris*, **250**, 3408–3410.

Martens, P. (1946). Cycle de dévelopment et sexualité des ascomycètes. *Cellule*, **50**, 125–310.

Pontecorvo, G. & Käfer, E. (1958). Genetic analysis based on mitotic recombination. *Advanc. Genet.* **9**, 71–104.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advanc. Genet.* **5**, 141–238.

Rizet, G., Lefort, C., Engelmann, N., Lissouba, P. & Mousseau, J. (1960). Sur un ascomycète intéressant pour l'étude de certains aspects du problème de la structure du gène. *C. R. Acad. Sci., Paris*, **250**, 2050–2052.

Roper, J. A. (1952). Production of heterozygous diploids in filamentous fungi. *Experientia*, **8**, 14–15.

Siddiqi, O. H. (1962). Mutagenic action of nitrous acid on *Aspergillus nidulans*. *Genet. Res.* **3**, 303–314.

Sturtevant, A. H. (1920). The vermilion gene and gynandromorphism. *Proc. Soc. exp. Biol., N.Y.*, **17**, 70–71.