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Segregation of factors controlling fusion between plasmodia of the true slime mould *Physarum polycephalum*

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1. INTRODUCTION

The life-cycle of the true slime mould (Myxomycete), *Physarum polycephalum* Schw., comprises two alternating phases, a macroscopic, multinucleate, syncytial plasmodium and small uninucleate amoebae. The plasmodium gives rise to spores which hatch to give the haploid amoebae, meiosis having taken place during spore formation. The amoebae segregate for 'mating type'. Fusion of amoebae of different mating type is necessary to initiate the diploid plasmodial phase. Four mating types controlled by four alleles (mt_1–mt_4) of a single gene have been demonstrated (Dee, 1966*a*). Amoebae carrying any one of the alleles will fuse with amoebae carrying any other to give plasmodia.

Fusion also occurs between plasmodia, but in this case genetic similarity is the prerequisite. The plasmodium is a motile syncytium of fluid form, showing vigorous, oscillating protoplasmic streaming. It spreads rapidly by growth and migration over the surface of agar medium. Pieces cut from plasmodia and placed on the same plate therefore meet after a few hours. Pieces cut from the same plasmodium fuse immediately after meeting, and massive streaming of protoplasm is visible between them. Bearing in mind the observation of Gray (1945) that plasmodial fusion does not occur between distantly related strains of *P. polycephalum*, we tested a number of plasmodia of different genotype for fusion. It was found that the ability to fuse segregated even among the progeny of a single plasmodium. The present paper reports experiments designed to investigate the genetic basis of plasmodial fusion, using closely related groups of plasmodia. An account of some preliminary studies was published as part of an earlier communication (Carlile & Dee, 1967). The model suggested to explain the results is that identity of plasmodial genotype at a single locus (f) is sufficient to allow fusion and that four alleles of f have been identified. Possible physiological inferences from the results are discussed.

Our results differ in several respects from those of Alexopoulos & Zabka (1962), Collins (1966) and Collins & Clark (1966), who studied plasmodial fusion in *Didymium iridis*.

2. MATERIALS AND METHODS

Strains. The strains of *P. polycephalum* used in our investigation were derived from two sources. The 'Wisconsin' amoebal clones *A7* and *i* were derived from a

plasmodium supplied by Dr H. P. Rusch of the University of Wisconsin in 1957. The 'Indiana' amoebal clones *B173* and *B174* were hatched from '*B17*' spores, derived from a plasmodium originally collected in Indiana and supplied to us by Professor C. J. Alexopoulos of the University of Texas. The origin of all these clones has been described more fully in previous publications (Dee, 1966*a*, *b*).

Culture methods. Amoebae were maintained in two-membered culture with *Escherichia coli* and cloned by plating (Dee, 1966*a*, *b*). Plasmodia were produced by crossing clones of amoebae (Dee, 1966*a*, *b*). When small plasmodia appeared on the cross-plates, drops of a semidefined agar medium (SDM) were added to nourish them. The SDM was a slightly modified form of that published by Daniel & Baldwin (1964). The plasmodia were freed from *E. coli* by migration across acidified (pH 4·6) agar or SDM agar. The axenic plasmodia were then cultured on SDM agar plates. When required, spore formation was induced on these plates by exposure to light. Spore viability was 1–5 %, which is similar to the viability of spores produced by previous methods.

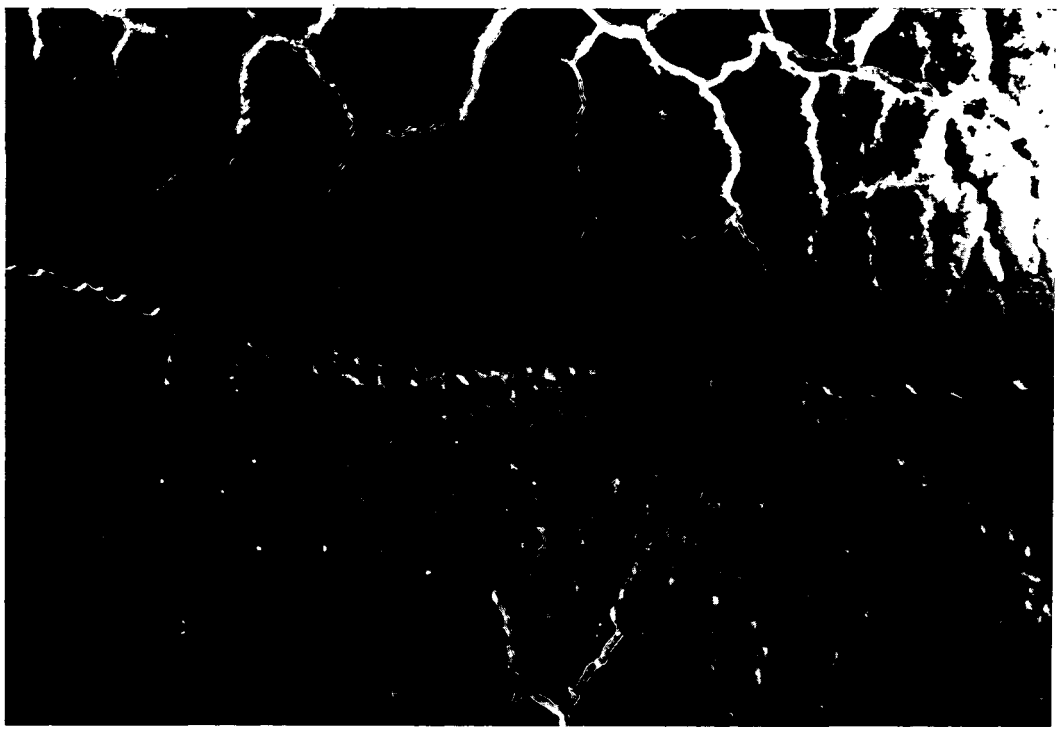
Fusion tests. To test the fusion behaviour of plasmodia, the method developed by Carlile & Dee (1967), or (more often) the following modification of it, was used. Plasmodia were inoculated onto a plate of half-strength SDM agar in the form of 1×2 cm blocks cut from plates showing vigorous plasmodial growth. The two blocks were placed 2 cm apart. The plasmodia migrated and grew onto the plate, meeting between 12 and 24 h after inoculation. Fusion was scored when protoplasm was seen to stream between plasmodia. In most experiments, the reaction between a pair of plasmodia could be scored unambiguously as 'fusion' or 'non-fusion' soon after they met, since fusion was almost immediate and large common veins quickly developed (Plate 1). Often the fused plasmodia later showed the lethal interaction described by Carlile & Dee (1967). In certain experiments, fusion was delayed and the lethal interaction occurred immediately after fusion, eliminating the small common veins which had developed. Since the small killed area was quickly overgrown, it was found necessary to observe the plasmodia in these tests continuously for 24 h in order to obtain unambiguous scoring. Observations in all experiments were made with a Wild M 5 stereomicroscope (magnifications $\times 6$, $\times 12$, $\times 25$, $\times 50$).

3. RESULTS

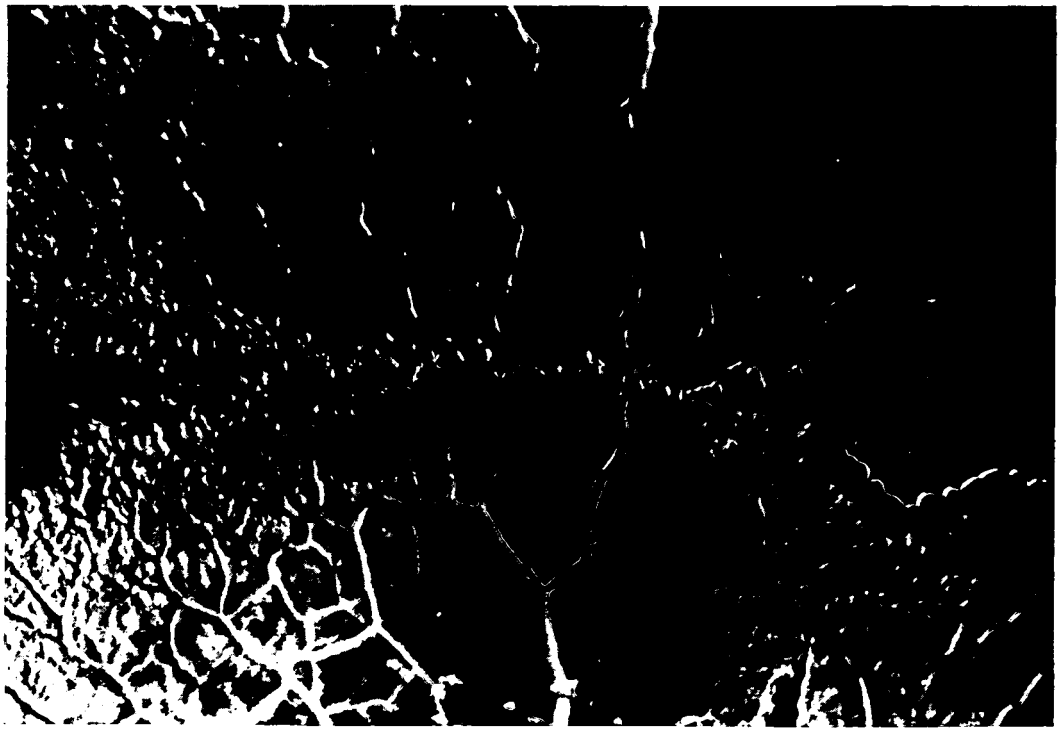
(i) *Wisconsin strain*

The clones of amoebae *A7* (mt_1) and *i* (mt_2) were crossed to give a plasmodium $A7+i$. Spores were obtained from $A7+i$ and 13 clones of mt_1 and 5 clones of mt_2 amoebae were isolated. These were crossed to the parent of opposite mating type ('backcrossed') and the resulting 18 plasmodia were tested for fusion with $A7+i$ (Table 1). Backcrossing was used so that part of the genotype of each plasmodium would be known.

Plasmodia of the four classes (I–IV) appearing in Table 1 were tested for fusion with one another. It was found that behaviourally only three classes were present, the two classes which fused with $A7+i$ (I and II) fusing freely with each other.



(*a*)



(*b*)

Plasmodia on SDM agar as they are seen in the experiments about 1 h after meeting. (*a*) No fusion: the plasmodia are in intimate contact; the 'veins' visible are not permanent structures but are simply the main channels of protoplasmic streaming (× 5). (*b*) Fusion: veins have been formed through which vigorous streaming is mixing the protoplasm of the two plasmodia (× 5).

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Plasmodia in classes III and IV fused only with members of the same class (Table 3).

The simplest scheme adequate to explain these results is that $A7+i$ is heterozygous for a pair of alleles (f_1 and f_2), that these alleles are segregating without

Table 1. *Results of testing plasmodia derived from the cross* A7 × i *for fusion with* A7 + i

(Plasmodia were produced by backcrossing progeny clones of $A7+i$ with the appropriate parent.)

Plasmodium				
Mating type of progeny clone	Mating type of parent clone	Reaction* with $A7+i$		
		Fusion	Non-fusion	Total
mt_1	mt_2 (strain i)	8 (I)	5 (III)	13
mt_2	mt_1 (strain $A7$)	2 (II)	3 (IV)	5

* Each result based on observation of at least four replicate tests.

Table 2. *Hypothetical* f*-types of plasmodia shown in Table 1*

Plasmodium			f-type deduced from reaction with $A7+i$ (f_1f_2)	
Genotype of progeny clone		Genotype of parent clone	Fusion	Non-fusion
mt_1f_1 mt_1f_2	}	mt_2f_2 (strain i)	f_1f_2 (I)	f_2f_2 (III)
mt_2f_1 mt_2f_2	}	mt_1f_1 (strain $A7$)	f_2f_1 (II)	f_1f_1 (IV)

Table 3. *Results of testing plasmodia of classes I–IV shown in Table 1 for fusion with one another*

(F = fusion; NF = non-fusion; f_1, f_2 are hypothetical alleles controlling fusion (see text).)

	I (f_1f_2)	II (f_2f_1)	III (f_2f_2)	IV (f_1f_1)
I (f_1f_2)	F	F	NF	NF
II (f_2f_1)	—	F	NF	NF
III (f_2f_2)	—	—	F	NF
IV (f_1f_1)	—	—	—	F

showing linkage to the mating-type (*mt*) locus, and that plasmodia must carry identical *f* alleles for fusion to be possible between them. Arbitrarily, *A7* is said to carry f_1 and *i* to carry f_2. Table 2 is an interpretation of Table 1 on the basis of this hypothesis. The *f* types assigned to the classes in this interpretation are consistent with the results of testing the classes against one another, as shown in Table 3.

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(ii) *Indiana strain*

The amoebal clones *B173* (mt_3) and *B174* (mt_4) were crossed and 13 progeny clones of mt_3 and 14 clones of mt_4 were isolated and backcrossed to the appropriate parent. Some of the resulting plasmodia were tested for fusion with *B173* + *B174* (Table 4). As in the similar test with the Wisconsin strain (Table 1), four classes of plasmodia were found. By the same reasoning as before, hypothetical *f* types were

Table 4. *Results of testing plasmodia derived from the cross* B173 × B174 *for fusion with* B173 + B174

(Plasmodia were produced by backcrossing progeny clones of *B173* + *B174* with the appropriate parent.)

Plasmodium		Reaction with *B173* + *B174* (f_4f_3)*		
Mating type of progeny clone*	Mating type of parent clone*	Fusion	Non-fusion	Total
mt_3f_3 mt_3f_4	mt_4f_3 (strain *B174*)	3 (f_4f_3)	2 (f_3f_3)	5
mt_4f_3 mt_4f_4	mt_3f_4 (strain *B173*)	3 (f_3f_4)	2 (f_4f_4)	5

* Hypothetical *f*-types are included (see text).

Table 5. *Results of testing plasmodia derived from the cross* B173 × B174 *against three tester strains*

(Plasmodia were produced by backcrossing progeny clones of *B173* + *B174* with the appropriate parent.)

Plasmodium							
Mating type of progeny clone	Mating type and *f*-type of parent clone	Reaction with tester strains			Deduced *f*-type of plasmodium	Deduced genotype of progeny clone	Total
		f_3f_3	f_4f_4	f_3f_4			
mt_3	mt_4f_3	F	F	NF	f_3f_3	mt_3f_3	7
mt_3	mt_4f_3	NF	NF	F	f_4f_3	mt_3f_4	6
mt_4	mt_3f_4	NF	NF	F	f_3f_4	mt_4f_3	6
mt_4	mt_3f_4	F	F	NF	f_4f_4	mt_4f_4	8

assigned to these classes (Table 4), the segregating alleles being termed f_3 and f_4, arbitrarily assigned to *B174* and *B173* respectively. Evidence for the allelism of f_3 and f_4 with f_1 and f_2 is presented below (3. iv). Three plasmodia representing the genotypes f_3f_3, f_4f_4, f_3f_4 were then used as tester strains to classify all the plasmodia from the cross *B173* × *B174* (Table 5). Those plasmodia fusing with the f_3f_4 tester were deduced to be either f_3f_4 or f_4f_3, depending on the parent clone used to produce them. This is consistent with the model requiring identity of *f*-type for fusion. On the same model, plasmodia failing to fuse with f_3f_4 were deduced to be f_3f_3 or f_4f_4, depending on the parent clone used. Contrary to expectation,

however, these plasmodia all fused with both f_3f_3 and f_4f_4 testers. In a further test, three of the assumed f_3f_3 and six of the assumed f_4f_4 plasmodia were tested against one another and were found to fuse in all combinations. These unpredicted fusions of f_3f_3 with f_4f_4 plasmodia can, we consider, be accommodated in the model (see Discussion).

(iii) *Tests showing that the behaviourally identical* f_3f_3 *and* f_4f_4 *plasmodia carry different* f *factors*

From the progeny amoebae of *B173* + *B174*, three clones of assumed genotype mt_3f_3 and three of mt_4f_4 were crossed in all possible combinations. These had, in the previous backcross experiment (3. ii, Table 5), produced the f_3f_3 and f_4f_4 plasmodial classes. The nine resulting plasmodia fused with an f_3f_4 tester and did not fuse with f_3f_3 and f_4f_4 testers, and were therefore classified as f_3f_4. This result confirmed the assumed genotypes of amoebal clones and demonstrated that the f_3f_3 and f_4f_4 plasmodia carried different f alleles.

(iv) *Allelism of* f_1 *and* f_2 *with* f_3 *and* f_4

The amoebal clones *A7* (mt_1f_1), *i* (mt_2f_2), *B173* (mt_3f_4) and *B174* (mt_4f_3) were crossed in all combinations. No fusions occurred between the resulting plasmodia, demonstrating that the factors segregating in the Wisconsin strains (*A7*, *i*) were not identical with the factors segregating in the Indiana strains (*B173*, *B174*). To test whether the two pairs of segregating factors (f_1, f_2 and f_3, f_4) were allelic,

Table 6. *Results of testing plasmodia derived from the cross* i × B174 *for fusion with* i + B174

Plasmodium: Mating type of progeny clone†	Plasmodium: Mating type of parent clone†	Reaction* with *i* + *B174* (f_2f_3)†: Fusion	Reaction* with *i* + *B174* (f_2f_3)†: Non-fusion	Total
mt_2f_2	mt_4f_3 (strain *B174*)	2 (f_2f_3)	5 (f_3f_3)	7
mt_2f_3				
mt_4f_2	mt_2f_2 (strain *i*)	8 (f_3f_2)	12 (f_2f_2)	20
mt_4f_3				

* Each result based on observation of two replicate tests. Plasmodia were produced by backcrossing progeny clones of *i* + *B174* with the appropriate parent.

† Hypothetical *f*-types included.

progeny of the cross *i* × *B174* were analysed. Seven clones of mt_2 and twenty clones of mt_4 were backcrossed to the appropriate parent and the resulting plasmodia tested for fusion with *i* + *B174* (Table 6). All the plasmodia were then tested against one tester strain from each of the four classes (Table 7). Assuming allelism of f_2 with f_3, these had the genotypes f_2f_2, f_3f_3, f_2f_3, f_3f_2 respectively. The plasmodia fell into three behavioural classes: twelve fusing with f_2f_2, five with f_3f_3, and ten with f_2f_3 and f_3f_2.

The results were consistent with the *f*-types deduced from the reactions of the

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plasmodia with $i + B174$. The ratio of $f_2 : f_3$ among the progeny was 14 : 13 (Table 7), which is consistent with the assumption of allelism of f_2 and f_3.

A sample of f_2f_2 and of f_3f_3 and all the f_2f_3 and f_3f_2 plasmodia were also tested against one another in all combinations. Fusion occurred in every test in which it was expected, but there was some delay in fusion between f_2f_3 and f_3f_2 strains (see Discussion).

A sample of f_2f_2 plasmodia from the cross $i \times B174$ was also tested against f_2f_2, f_1f_2 and f_1f_1 plasmodia derived from the cross $A7 \times i$ and was found to fuse only with the f_2f_2 class.

Table 7. *Results of testing plasmodia derived from the cross* i × B174 *against four tester strains*

(The plasmodia (including the tester strains) are those appearing in Table 6.)

Plasmodium: Mating type of progeny clone	Plasmodium: Mating type and *f*-type of parent clone	Reaction with tester strains: f_2f_2	f_3f_3	f_2f_3	f_3f_2	Deduced *f*-type of plasmodium	Deduced genotype of progeny clone	Total
mt_2	mt_4f_3	NF	NF	F	F	f_2f_3	mt_2f_2	2
mt_2	mt_4f_3	NF	F	NF	NF	f_3f_3	mt_2f_3	5
mt_4	mt_2f_2	F	NF	NF	NF	f_2f_2	mt_4f_2	12
mt_4	mt_2f_2	NF	NF	F	F	f_3f_2	mt_4f_3	8

Table 8. *Tests of plasmodia produced by crossing progeny clones of* B173 + B174 *with* i

(The clones used were a sample of the four genotypes appearing in Table 5.)

Plasmodium: Genotype of progeny clone*	Plasmodium: Genotype of *i*	Reaction with testers: *i* + *B174* ($mt_2f_2 + mt_4f_3$)	*i* + *B173* ($mt_2f_2 + mt_3f_4$)
mt_3f_3	mt_2f_2	F	NF
mt_3f_4	mt_2f_2	NF	F
mt_4f_3	mt_2f_2	F	NF
mt_4f_4	mt_2f_2	NF	F

* Three clones of each genotype were used.

(v) *Further analysis of the* B173 × B174 *progeny making use of the* f_2 *allele*

From the cross *B173* × *B174*, three progeny clones of each of the assumed genotypes, mt_3f_3, mt_3f_4, mt_4f_3 and mt_4f_4, were crossed with i (mt_2f_2). The resulting plasmodia were tested for fusion with $i + B174$ (f_2f_3) and $i + B173$ (f_2f_4). The plasmodia fused with either one or the other tester strain (Table 8), indicating that only two allelic factors (f_3 and f_4) were segregating among the progeny of the *B173* × *B174* cross and gave rise to the behavioural classes observed in Table 4.

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4. DISCUSSION

The following model is proposed to account for the results. Plasmodial fusion in the strains of *P. polycephalum* studied is controlled by four alleles (f_1–f_4) of a single gene *f*. With the exception of the fusions between f_3f_3 and f_4f_4 plasmodia (discussed below), fusion is possible only between plasmodia carrying the same *f* alleles. The mating-type locus apparently does not affect fusion behaviour, since some plasmodia carrying the same *mt* alleles fail to fuse (e.g. Table 1) and some plasmodia carrying different *mt* alleles fuse (Table 8). The *f* locus is unlinked to the *mt* locus, as shown by the equal numbers of recombinant and parental types amongst the progeny of each cross (e.g. Table 5).

Although fusion in the strains studied is under the control of a single gene, it appears that the rate of fusion is influenced by other genes. In the cross between the distantly related strains *i* and *B174*, fusion between plasmodia of the types f_2f_3 and f_3f_2 was unusually delayed, the plasmodia remaining in contact for up to 24 h before fusing. Since fusion of these plasmodia with others in the same class was not delayed and since the classes were made by up crossing progeny clones of *i* + *B174* with *B174* or *i* respectively, it is likely that the delay was caused by genetic dissimilarities in the parental component. It is concluded that modifying genes are operating which discourage fusions between dissimilar strains.

(i) *Fusions between* f_3f_3 *and* f_4f_4 *plasmodia*

The experiments reported in section 3 (iii) demonstrated that the f_3f_3 and f_4f_4 plasmodia, although identical in fusion behaviour, in fact carried different *f* alleles. The experiments reported in 3 (v), in which only two classes of plasmodia were found when progeny clones of *B173* + *B174* were crossed with an f_2 strain, showed that only two *f* factors (f_3 and f_4) were segregating amongst the progeny. This confirmed that the four plasmodial classes amongst the backcrossed progeny of *B173* + *B174* were f_3f_3, f_4f_4, f_3f_4 and f_4f_3.

The heterozygotes f_3f_4 and f_4f_3 behave as expected throughout, as do f_2f_3 and f_2f_4. The heterokaryon resulting from fusion between f_3f_3 and f_4f_4 plasmodia behaves, after a short delay, as an apparent f_3f_4 (Poulter, unpublished). The heterokaryon is, however, unstable and reverts eventually to parental type. The unusual behaviour of the f_3 and f_4 alleles is thus confined to the homozygotes.

The model proposed (Poulter, unpublished) to explain the fusion of the f_3f_3 and f_4f_4 plasmodia is as follows. The genotype of f_3 and f_4 amoebal clones is in some way defective, the f_3 clones carrying one defect and the f_4 clones another. The defects could be in *f* or in another locus but the evidence suggests that they are either in *f* or in a closely linked locus (see below). In the homozygotes (f_3f_3 or f_4f_4) these defects are expressed, while in the heterozygote f_3f_4 they complement each other. Since the 'defective' homozygotes show unexpected fusions, their defect must be in a process which normally prevents fusion between dissimilar plasmodia. The model therefore requires that the complementing factors act in a

process which inhibits fusion between dissimilar strains. Since identity of *f* factors is not necessary for fusion, the *f* factors are presumably not acting as recognition or catalysing factors but are an integral part (or possibly the whole) of the process which prevents the fusion between dissimilar strains. If they were recognition factors or catalysts of fusion, it would not be possible to explain the exceptional results in terms of defectively functioning homozygotes, since defective functioning of the factors would result in unexpected *non-fusions*.

If the complementation in the f_3f_4 heterozygote is due to two or more loci, recombinant classes of amoebae should occur in the progeny of an f_3f_4 plasmodium. When backcrossed these recombinant classes would give, for example, f_3f_3 plasmodia showing complementation, which would not fuse with f_4f_4 testers. No such recombinant classes have been found amonst 37 progeny clones analysed. The complementing sites are therefore closely linked to each other and to the *f* locus (which may in fact be one of the complementing sites) or both are alleles of the *f* locus.

If intragenic complementation is operating, the action of the *f* gene probably involves the production of dimers (or higher polymers). Since recognition of *f* type is most likely to occur at the surface of two plasmodia in contact, it is here that the dimers may occur. It is supposed that this surface barrier of dimers inhibits fusion of a plasmodium with any other plasmodium with which it may be in contact, unless the barrier is cancelled by identical dimers carried by the other plasmodium. When two plasmodia showing deficient barriers (e.g. an f_3f_3 and f_4f_4) meet, they fuse. When a deficient plasmodium meets a plasmodium carrying an inhibitory coat (e.g. an f_3f_3 meets an f_3f_4), the barrier remains uncancelled and fusion cannot occur. The cancelling process could be envisaged as the polymerization of identical dimers to give tetramers. If this model for the action of the *f* gene is correct, it presents interesting parallels and contrasts with the suggested mode of action of the *S* (self-incompatibility) gene in some higher plants (Lewis, 1964). Lewis proposed that the *S* gene produces dimers which exist in both pollen and style. Self-pollination brings together tissues carrying identical dimers, which combine to give a tetramer inhibitory to pollen tube growth. In the proposed model for the *f* system the dimers are the biologically active form, inhibiting fusion, and the formation of tetramers removes this inhibition. A physiological test for the model is being attempted.

(ii) *Ecological implications*

The *f* gene is a mechanism discouraging the formation of heterokaryons by preventing fusion between distantly related plasmodia and reducing the chance of fusion between plasmodia of the same population. The frequency of heterokaryons in a natural population will be in inverse proportion to the number of *f* alleles carried by that population. The populations from which our two original plasmodia were isolated may well have carried more than two pairs of alleles. The operation of the *f* gene and the killing reaction which often follows fusion (Carlile & Dee, 1967) must result in heterokaryons being infrequent in natural populations.

Caten & Jinks (1966), considering the action of similar factors in the fungi, have also concluded that heterokaryosis is rarer in natural populations than previously supposed.

SUMMARY

1. The occurrence of fusion between plasmodia produced from amoebal clones of *P. polycephalum* was studied.

2. The occurrence of fusion was found to be strain-dependent and the factors responsible segregated in the progeny of a cross.

3. The segregations found in crosses between several strains led to the conclusion that four alleles (f_1–f_4) of one gene *f* were controlling fusion in these strains.

4. Fusion occurs only between plasmodia carrying identical *f* alleles, except in one class of results.

5. A model accommodating all the results, including the 'exceptional' class, is proposed. It requires that the action of the *f* factors is to inhibit fusion between dissimilar strains rather than to promote fusion between identical strains. Certain physiological deductions from this model are discussed.

6. The locus (*mt*) determining mating type of the amoebae is not concerned in plasmodial fusion and is unlinked to *f*.

7. The rate of fusion between some pairs of strains is apparently influenced by modifying genes.

8. It is suggested that, as a result of the operation of the *f* gene and of the previously described killing reaction, heterokaryons will occur rarely in natural populations of *P. polycephalum*.

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