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Genetic analysis of a cross between two homothallic strains of *Physarum polycephalum*

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SUMMARY

The homothallic amoebal clones of *Physarum polycephalum* are of potential use in understanding the developmental genetics of this organism. Such an application requires that complementation and recombination analysis be possible between pairs of homothallic clones. This paper is a report of the formation of mixed plasmodia by pairs of homothallic amoebal clones. In order to detect such mixed plasmodia use was made of two marker genes involved in plasmodial fusion, *fus*A and *fus*B. Sporulation of a mixed plasmodium formed from two homothallic (delayed) amoebal clones yielded progeny amoebae which were genetically recombinant. It is deduced from the ratios of various genotypes in these progeny clones that the mixed plasmodium was diploid and that meiosis was associated with sporulation. There is therefore no impediment to the use of the homothallic strains for genetical analysis. The progeny amoebal clones were observed to be showing segregation for the characters homothallic (rapid) and homothallic (delayed). This observation, taken together with other related observations, suggests that the homothallic (delayed) character is produced by mutation of the homothallic (rapid) character. The rare plasmodia formed by a homothallic (delayed) amoebal clone are the result of reversion of this mutation. Amoebal clones of the homothallic (delayed) type are therefore developmental mutants unable to perform the differentiation from amoeba to plasmodium.

1. INTRODUCTION

The value of the Myxomycete *Physarum polycephalum* for the study of the regulation of developmental processes and the work of various groups in this field has been recently reviewed (Dee, 1975). There is now a sound basis on which genetical analysis of this organism can proceed.

Prior to 1970, only heterothallic strains, in which haploid amoebae of differing mating type fused to form a diploid plasmodium, had been described. Such a heterothallic system was unsuitable for the genetical analysis of plasmodial characters, or for the genetical analysis of the process of plasmodial formation because of the difficulty of obtaining homozygous mutant diploid genotypes. This difficulty was circumvented by the work of Wheals (1970) who showed that amoebae of the Colonia isolate of *P. polycephalum* produced plasmodia within

clones. Genetic analysis by Wheals of the progeny of crosses between the Colonia strain *C*50 and heterothallic amoebae showed that the ability of *C*50 amoebae to produce plasmodia within clones was apparently due to a single allele at the mating-type (*mt*) locus which he designated mt_h. Cooke & Dee (1974) reported measurements of nuclear DNA content of mt_h amoebae and clonally produced plasmodia and claimed that there is no change in ploidy when mt_h amoebae form plasmodia, the plasmodia remaining haploid. The advantages of the mt_h amoebae for genetical analysis are obvious, and were the reason for Wheals' investigation of the system. The original homothallic strain *C*50 only rarely formed plasmodia. Cooke & Dee (1975) reported that spores produced from a *C*50 plasmodium germinate to give clones of amoebae which form plasmodia much more readily. They designated one such clone *CL* and this strain has been employed extensively in genetic analysis. *CL* amoebae form plasmodia in very small amoebal plaques. Cooke & Dee (1975) also reported that following repeated subculture subclones of *CL* occurred which showed delayed plasmodium formation, closely resembling the original *C*50 type. Once such delayed clone was termed *CL*d.

In order to perform genetic analysis it is necessary to be able to combine the genotypes of two clones, that is to perform crosses. Cooke & Dee (1975) reported that they were unable to detect crosses between *CL* amoebae and heterothallic clones, but could demonstrate crossing between *CL*d clones and heterothallic clones. Use was made in this analysis of the fusion genes *fus*A and *fus*B as genetic markers. Cooke & Dee (1974) reported that plasmodia derived from clone *CL*d resemble clone *CL* plasmodia in that they have a haploid DNA content. Plasmodia produced by crossing clone *CL*d with heterothallic clones had the diploid DNA content. Analysis of the unlinked *fus*A and *fus*B genetic markers confirmed that such plasmodia were diploid. The work reported here is an investigation by means of the genetic markers *fus*A and *fus*B of plasmodia formed by crossing in pairs clones of the *CL*d type. In other words, amoebal clones of the mt_h (delayed) type, genetically distinguished by the *fus*A and *fus*B alleles they carried, were brought together and plasmodia of mixed genotype detected and analysed. The *fus*A and *fus*B loci are of special use in this system since not only are the alleles readily and unambiguously scored but the mixed plasmodia have a distinct fusion type which prevents them from fusing with plasmodia formed by selfing of the two amoebal clones. This enables mixed plasmodia to be detected and analysed without the confusion of an admixture of selfed nuclei. Unexpectedly the results clearly demonstrate that such mixed plasmodia are genetically diploid. It appears therefore that clones of the *CL*d type can either give rise to haploid plasmodia, or under certain circumstances, give rise to diploid plasmodia Haploid plasmodia could arise from amoebal clones of 'Colonia' either apogamically (without fusion of amoebae) or by coalescence (which was used by Cooke & Dee to designate a process characterized by amoebal fusion without subsequent karyogamy). The diploid plasmodia detected in this work indicate that amoebal clones of the 'Colonia' isolate may, as originally believed by Wheals, give rise homothallically to plasmodia. Homothallism is used in this context to describe the formation of a

zygote by the fusion of two cells derived asexually from a common parental cell. Such homothallic events are, we believe, rare in cultures of 'Colonia' and its derivatives. The use of genetic markers, for example *fus*A and *fus*B, is necessary to enable one to readily detect homothallically produced diploid plasmodia.

In order to avoid unnecessarily cumbersome description and confusion with respect to previous reports, the strains of *P. polycephalum* which form plasmodia within clones will, following the original description of Wheals (1970), be described in this report as homothallic. This is not intended to suggest that there is any doubt that Cooke & Dee (1974) are correct, most such plasmodia are the result not of a homothallic event but of an apogamic event. This analysis was a necessary preliminary to the use of these strains in genetical analysis.

2. MATERIALS AND METHODS

(i) *Strains.* The origin of the heterothallic amoebal strains *a*, *i*, *LU*648 and *LU*688 has been described previously (Dee, 1966; Cooke & Dee, 1975). Strains *LU*648 and *LU*688 are substantially isogenic with strain *CL* at all loci other than *mt* and *fus*A. The origin of the homothallic strains *CL* and *CL*d has been described previously (Wheals, 1970; Cooke & Dee, 1975). *OU*18 is a progeny clone of $a \times i$.

(ii) *Loci. mt*, mating type. Alleles mt_1, mt_2, heterothallic (Dee, 1966); mt_h plasmodial formation within amoebal clones, previously thought to be homothallic (Wheals, 1970), and later reinterpreted as apogamic (Cooke & Dee, 1974). The mt_h locus is found in two types of amoebae, those which form plasmodia rapidly in clones (e.g. *CL*) and those which show a delay (e.g. *CL*d).

*fus*A and *fus*B, plasmodial fusion type. Identity of *fus*A and *fus*B phenotype is a prerequisite for plasmodial fusion. The two alleles at *fus*A, *fus*A1 and *fus*A2 are codominant. Two alleles are known for *fus*B, of which *fus*B2 is dominant to *fus*B1 (Poulter, 1969; Cooke & Dee, 1975). Thus six fusion phenotypes result from the possible combinations of alleles at these loci.

sax$^+$ and *sax*$^-$, sensitivity to axenic medium. *Sax*$^-$ is recessive, *sax*$^-$ homozygotes die when transferred to SDM (Poulter, 1969).

(iii) *Genotypes of amoebal strains.* *i*:mt_2 *fus*A2 *fus*B2.*LU*648:mt_1*fus*A1 *fus*B1. *LU*688:mt_2 *fus*A1 *fus*B1.*CL*, *CL*d:mt_h *fus*A2 *fus*B1.*OU*18: mt_1*fus*A1 *fus*B2 *s ax*$^-$. (All strains used in this work other than *OU*18 are *sax*$^+$.)

(iv) *Cultural conditions.* Plasmodia were maintained at 26 °C on semi-defined medium, pH 4·6 (Dee & Poulter, 1970). Amoebae were maintained in two membered culture with *Escherichia coli* at 26 °C on 5% SDM (pH 7). Production of spores, spore plating and isolation of progeny clones were carried out as previously described (Wheals, 1970).

(v) *Plasmodium formation.* Amoebal clones were crossed, or permitted to form plasmodia within clones, on 5% SDM (pH 7). The amoebae were inoculated onto plates already spread with *Escherichia coli*. Plasmodia were removed from such plates on blocks of the agar medium, and inoculated onto 50% SDM (pH 4·6).

(vi) *Plasmodial fusion tests.* The methods of Poulter & Dee (1968) were employed. Plates of 50% SDM were inoculated with the unknown plasmodium to be classified together with a plasmodium of known fusion phenotype. The test was repeated with each of the six known fusion types. The unknown plasmodium invariably fused with one of the six testers, and failed to fuse with the other five. Fusion behaviour is scored after a period (usually 48 h) of growth on the SDM plate, during which time the pair of plasmodia make contact with each other.

Unless otherwise specified the fusion testers employed in this analysis were *LU*648 (*mt*$_1$ *fus*A1 *fus*B1) × *LU*688 (*mt*$_2$ *fus*A1 *fus*B1) to give fusion group I; *OU*18 (*mt*$_1$ *fus*A1 *fus*B2) × *LU*688 to give fusion group II; *LU*648 × *CL*d (*mt*$_h$ *fus*A2 *fus*B1) to give fusion group III; *LU*648 × *i* (*mt*$_2$ *fus*A2 *fus*B2) to give fusion group IV; *CL*d (selfed) to give fusion group V and *CL*d × *i* to give fusion group VI. These testers were easily constructed with the exception of *CL*d × *i* which is not readily formed. This failure of *mt*$_2$ clones to cross readily with *CL*d has already been noted (Cooke & Dee, 1975; Wheals, 1970).

3. RESULTS

(i) *Isolation of strain* OUh*3*. (mt$_h$ (*delayed*) fus*A1* fus*B2*).

The isolation of this strain containing the desired combination of genetic markers consisted of two steps. The initial step was the isolation of strain *OUg*3 (*mt*$_1$ *fus*A1 *fus*B2). The stock strain carrying these markers in our laboratory (*OU18*) also carries the *sax*$^-$ allele, and since the *sax*$^+$ allele was needed in this analysis *OU*18 could not be employed. In order to produce *OUg*3, clones *LU*648 (*mt*$_1$ *fus*A1 *fus*B1) and *i* (*mt*$_2$ *fus*A2 *fus*B2) were crossed and the plasmodium sporulated. Progeny amoebal clones were then isolated from the spore plaques. These 61 clones were tested for mating type by crossing with *LU*648, *LU*688 and *i* (Table 1). All the plasmodia formed from this test were subcultured onto SDM and fusion tested with the six-tester plasmodia of known fusion type (see Materials and Methods).

Amoebal clones of all 8 possible genotypes were found in the 61 progeny clones (Table 1). Amongst these were 9 of the desired type *mt*$_1$ *fus*A1 *fus*B2. One of these, designated *OUg3*, was used for the subsequent step in this isolation programme.

It is noteworthy that plasmodia corresponding to all 6 known fusion types were found in this analysis, and contrary to the suggestion of Olive (1975), based on the work of Collins (1972), there is no doubt that the *fus*A1/*fus*A2, heterozygote is expressed as a fusion phenotype totally distinct from either the *fus*A1/*fus*A1 homozygote, or the *fus*A2/*fus*A2 homozygote.

The next step in this preparation was the isolation of strain *OU*h3 (*mt*$_h$ (delayed) *fus*A1 *fus*B2). To perform this, clone *CL*d (*mt*$_h$ (delayed) *fus*A2 *fus*B1) was crossed with strain *OUg*3 (*mt*$_1$ *fus*A1 *fus*B2). As expected when this cross was performed two types of plasmodia were formed which failed to fuse with each other. One type of plasmodium was fusion group V, and could therefore be deduced to be derived by selfing of the homothallic delayed clone *CL*d (*fus*A2 *fus*B1). The

other type of plasmodium formed was fusion group IV, and could therefore be deduced to be a crossed plasmodium. A crossed (fusion group IV) plasmodium was sporulated, and the spores geminated. A 1:1 ratio of $mt_h : mt_1$ was, as expected, observed in these clones. All homothallic clones were of the delayed type. From amongst these mt_h clones one of type *fus*A2 *fus*B1 was selected and designated *OUh*3.

Table 1. *Analysis of progeny clones from the cross* LU*648* × i (mt_1 fusA_1 fusB_1 × mt_2 fusA_2 fusB_2)

	Fusion class of plasmodia from cross			
Genotype of clone	*LU*648 mt_1 *fus*A_1 *fus*B_1	*LU*688 mt_2 *fus*A_1 *fus*B_1	*i* mt_2 *fus*A_2 *fus*B_2	No. of clones
mt_1 *fus*A_1 *fus*B_1	No plasmodia	I	IV	7
fusA_1 *fus*B_2		II	IV	9
fusA_2 *fus*B_1		III	VI	4
fusA_2 *fus*B_2		IV	VI	12
				32
mt_2 *fus*A_1 *fus*B_1	I	No plasmodia	No plasmodia	6
fusA_1 *fus*B_2	II			11
fusA_2 *fus*B_1	III			6
fusA_2 *fus*B_2	IV			6
				29

(ii) *Production of a mixed plasmodium derived from clones of genotype* mt_h (*delayed*) fus*A1* fus*B2 and* mt_h (*delayed*) fus*A2* fus*B1*

Plasmodia of this type should be formed when clones *OUh*3 and *CL*d are mixed. However, such 'cross'-plates will also produce selfed plasmodia of the two types. The three types of plasmodium will be of distinct fusion phenotype, the mixed plasmodium being fusion group IV (*fus*A1/*fus*A2, *fus*B1/*fus*B2) and the selfed plasmodia of fusion groups II (*fus*A1 *fus*B2) or V (*fus*A2 *fus*B1).*OUh*3 and *CL*d were mixed on 10 plates and on each plate a number of plasmodia were produced. The agar medium of the cross-plates was quartered, and all the quadrants each carrying several plasmodia were transferred to SDM. Following incubation of such plates usually several large non-fusing plasmodia were present. These plasmodia would be produced not simply by growth but also by fusion of isogenic plasmodia. In this way a large number of separate plasmodia could be analysed, by first allowing them to group themselves into the several fusion types present. Inocula were taken from each of the non-fusing plasmodia on these plates. 54 such plasmodia were subcultured and fusion tested, 25 were of fusion group II (selfed *OUh*3), 28 were of fusion group V (selfed *CL*d), and one was of mixed type (fusion group IV).

(iii) *Analysis of progeny clones from the mixed plasmodium* OUh*3* × CL*d*

The plasmodium *OUh*3 × *CL*d was sporulated and 183 amoebal clones isolated (Table 2). All these clones produced plasmodia; confirming that both of the parental types were mt_h. One hundred and ten of these clones formed plasmodia rapidly in plaques (resembling *CL*) while 73 formed plasmodia rarely and after a delay (resembling *OUh*3 and *CL*d). This result, the appearance of the rapid homothallic character at this point in the analysis was unexpected.

Table 2. *Analysis of progeny clones from the cross* OUh*3* × CL*d* (mt_h fusA_1 fusB_2 × mt_h fusA_2 fusB_1)

Genotype of clone	Fusion class of selfed clone	Plasmodial formation	No. of clones	Totals
mt_h *fus*A_1 *fus*B_1	I	Delayed	11	38
		Rapid	27	
mt_h *fus*A_1 *fus*B_2	II	Delayed	16	43
		Rapid	27	
mt_h *fus*A_2 *fus*B_1	V	Delayed	21	44
		Rapid	23	
mt_h *fus*A_2 *fus*B_2	VI	Delayed	18	49
		Rapid	31	

One hundred and seventy-four clones were selected for further analysis, and within these clones plasmodia were allowed to form and the resulting plasmodia were fusion tested. Amongst the plasmodia formed by these 174 clones no fusion type III or IV plasmodia were found, which supports the belief that the plasmodia were clonally derived from haploid amoebae (both III and IV plasmodia must be heterozygous for *fus*A). Plasmodia representing each of the other fusion groups were found (Table 2). The ratio of the four types in both the rapid homothallic and delayed homothallic group strongly suggests a 1:1:1:1 ratio. This is most simply interpreted as indicating that the mixed plasmodium *OUh*3 × *CL*d was diploid, and that recombination had occurred between the unlinked loci *fus*A and *fus*B in a doubly heterozygous parental plasmodium.

A killing reaction never occurs when isogenic plasmodia fuse. A majority of the group II progeny plasmodia gave killing when fused with a plasmodium derived by selfing from parental clone *OUh*3. A majority of the group V progeny plasmodia gave killing when fused with a plasmodium derived from parental clone *CL*d. This is further evidence that these progeny clones were not isogenic with the parental types and that therefore recombination must have occurred at some point. (The genetics of the killing reaction are being analysed further using this system.) The genetics of the killing reaction in some strains of *P. polycephalum* is now understood (Carlile, 1976).

4. DISCUSSION

Wheals (1970) originally suggested that those amoebal strains of *P. polycephalum* which formed plasmodia within clones were doing so by a homothallic event, and that such plasmodia were therefore diploid. Cooke & Dee (1974), on the basis of DNA content of nuclei, demonstrated that in fact all such plasmodia that they analysed were haploid. This report is a genetical investigation of the state of plasmodia derived from mt_h amoebal strains. In order to do this a plasmodium was produced by mixing two mt_h (delayed) clones carrying appropriate genetic markers. The progeny cloned from spores of such a plasmodium show free recombination between the genetic markers *fus*A and *fus*B. Therefore the nuclei from the two original amoebal clones must have fused. There is therefore no possibility that the mixed plasmodium *OUh*3 × *CL*d was a haploid heterokaryon which sporulated without meiosis.

The only likely explanation compatible with the 1:1:1:1 ratios found in the progeny of this plasmodium is that the plasmodium *OUh*3 × *CL*d was a doubly heterozygous diploid. In order to detect recombination more than one locus must be heterozygous, and since mt_h must be homozygous in this analysis the most suitable choice of genetic markers was *fus*A and *fus*B.

If, using these markers, diploid plasmodia can be found derived from mt_h clones, then it is also probable that within a pure clone of mt_h type, diploid plasmodia are on occasion produced. This event is, however, no doubt a rarity in a pure clone, since it was found to be rare in the mixed clone situation. Therefore amoebal strains of the mt_h type are probably best described as having an ability to form haploid plasmodia apogamically, and an ability to form infrequent diploid plasmodia homothallically.

It is of interest that in this work, and in numerous subsequent experiments of a similar type, no evidence has been found of the derivation from mt_h clones of mixed plasmodia which are genetically haploid heterokaryons. We consider that such plasmodia, which must arise as intermediates in the formation of mixed diploids, are probably genetically unstable. We believe that if they do not proceed to diploidy then they become unbalanced during mitotic growth and come to resemble one or other of the selfed plasmodial types, losing their mixed fusion phenotype. Evidence supporting this belief will be provided in a subsequent report.

The appearance of the rapid homothallic character in the progeny of the plasmodium *OUh*3 × *CL*d requires some explanation (both *OUh*3 and *CL*d were delayed homothallics). The ratio of rapid to delayed mt_h alleles in the *OUh*3 × *CL*d progeny clones approximates 1:1. Cooke & Dee (1975) summarized some information relevant to this problem. They reported that when *CL*d was crossed with heterothallic clones and the (diploid) plasmodium sporulated the progeny clones showed mt_h (delayed) or heterothallic characters, and that these occurred in the ratio 1:1. This observation, which is confirmed by the present analysis of *OUh*3 × *CL*d, suggests that the mt_h (delayed) character can pass unchanged through the meiotic cycle, behaving as an allele of the *mt* locus. Cooke & Dee (1975) also

reported that if the plasmodia formed within an amoebal clone of the homothallic delayed type are sporulated, the progeny clones are all of the rapid homothallic type. Taken together with our present observations we suggest that the data supports the hypothesis that the mt_h (delayed) character is a mutant form of the mt_h (rapid) character. This mutant form is stable through the meiotic cycle. However, during growth of a clone of the mt_h (delayed) type revertants can occur back to the original rapid homothallic character. It is such revertant events which give rise to the rare 'delayed' plasmodia found in such clones. Such plasmodia if sporulated are found, as expected on this hypothesis, to give rise to only rapid homothallic progeny. Thus we suggest a clone of type mt_h (delayed) cannot give rise to plasmodia unless it has reverted to mt_h (rapid). This hypothesis would explain the appearance of the rapid homothallic character in the present analysis. A mixed culture of clones *OUh3* (mt_h delayed) and *CL*d (mt_h delayed) would be unable to give rise to the diploid plasmodium *OUh3* × *CL*d unless one or other of the clones reverted to the mt_h (rapid) type. Following such a reversion of an amoeba of one clone or the other the resulting mixed plasmodium would be heterozygous for the characters mt_h (rapid) and mt_h (delayed). The progeny clones would, as reported, show a 1:1 segregation for this character.

If this is correct then *OUh3* × *CL*d can be viewed as not simply a double heterozygote (for *fus*A1/*fus*A2, and *fus*B1/*fus*B2) but a triple heterozygote involving also mt_h (rapid) mt_h (delayed). This hypothesis suggests that the mt_h (rapid) character is dominant to the mt_h (delayed) character. In the cross mt_h (delayed) × heterothallic, rapid plasmodial formation occurs which supports the suggestion that the delayed character is recessive. Analysis of our data shows, as expected, no linkage between the mt_h types and the fusion loci; in other words the plasmodium is behaving as a normal diploid with respect to this triple heterozygous state.

An interesting question concerns whether the mt_h (delayed) character is an allelic form of the mt_h (rapid) character, and whether these are truly allelic to the *mt* locus as defined in heterothallic strains. A subsequent report will present evidence to support our belief that this is a complex locus containing several complementation groups. However, in this report we have retained the terminology of referring to mt_h (rapid) and mt_h (delayed) characters as if they were truly alleles of the *mt* locus as defined in heterothallic systems.

The purpose of this work was to determine whether the strains of *P. polycephalum* which give rise to plasmodia within clones were capable of being used not only in studies based on mutagenesis, but also in subsequent complementation and recombination analysis. This report makes clear that since they are capable of forming diploids which can be detected by appropriate genetic markers, there is no impediment to their use for complementation and recombination analysis.

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