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, *fusA* and *fusB*. 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 C50 and heterothallic amoebae showed that the ability of C50 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 C50 only rarely formed plasmodia. Cooke & Dee (1975) reported that spores produced from a C50 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 C50 type. Once such delayed clone was termed CLd.

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 CLd clones and heterothallic clones. Use was made in this analysis of the fusion genes fusA and fusB as genetic markers. Cooke & Dee (1974) reported that plasmodia derived from clone CLd resemble clone CL plasmodia in that they have a haploid DNA content. Plasmodia produced by crossing clone CLd with heterothallic clones had the diploid DNA content. Analysis of the unlinked fusA and fusB genetic markers confirmed that such plasmodia were diploid. The work reported here is an investigation by means of the genetic markers fusA and fusB of plasmodia formed by crossing in pairs clones of the CLd type. In other words, amoebal clones of the mt_h (delayed) type, genetically distinguished by the fusA and fusB alleles they carried, were brought together and plasmodia of mixed genotype detected and analysed. The fusA and fusB 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 CLd 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
Genetic analysis of Physarum

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 fusA and fusB, 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, LU648 and LU688 has been described previously (Dee, 1966; Cooke & Dee, 1975). Strains LU648 and LU688 are substantially isogenic with strain CL at all loci other than mt and fusA. The origin of the homothallic strains CL and CLd has been described previously (Wheals, 1970; Cooke & Dee, 1975). OU18 is a progeny clone of a×i.

(ii) Loci. mt, mating type. Alleles mt1, mt2, heterothallic (Dee, 1966); mtb plasmodial formation within amoebal clones, previously thought to be homothallic (Wheals, 1970), and later reinterpreted as apogamic (Cooke & Dee, 1974). The mtb 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. CLd).

fusA and fusB, plasmodial fusion type. Identity of fusA and fusB phenotype is a prerequisite for plasmodial fusion. The two alleles at fusA, fusA1 and fusA2 are codominant. Two alleles are known for fusB, of which fusB2 is dominant to fusB1 (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:mt2 fusA2 fusB2.LU648:mt1 fusA1 fusB1. LU688:mt2 fusA1 fusB1 CL, CLd:mtb fusA2 fusB1.OU18: mt1 fusA1 fusB2 sax−. (All strains used in this work other than OU18 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

- LU648 (mt fusA1 fusB1) × LU688 (mt fusA1 fusB1) to give fusion group I;
- OU18 (mt fusA1 fusB2) × LU688 to give fusion group II;
- LU648 × CLd (mt fusA2 fusB1) to give fusion group III;
- LU648 × i (mt fusA2 fusB2) to give fusion group IV;
- CLd (selfed) to give fusion group V and CLd × i to give fusion group VI.

These testers were easily constructed with the exception of CLd × i which is not readily formed. This failure of mt2 clones to cross readily with CLd has already been noted (Cooke & Dee, 1975; Wheals, 1970).

3. RESULTS

(i) **Isolation of strain OUh3.** (mt fusA1 fusB2).

The isolation of this strain containing the desired combination of genetic markers consisted of two steps. The initial step was the isolation of strain OUg3 (mt fusA1 fusB2). 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 OU18 could not be employed. In order to produce OUg3, clones LU648 (mt fusA1 fusB1) and i (mt fusA2 fusB2) 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 LU648, LU688 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 fusA1 fusB2. 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 fusA1/fusA2 heterozygote is expressed as a fusion phenotype totally distinct from either the fusA1/fusA1 homozygote, or the fusA2/fusA2 homozygote.

The next step in the preparation was the isolation of strain OUh3 (mt (delayed) fusA1 fusB2). To perform this, clone CLd (mt delayed fusA2 fusB1) was crossed with strain OUg3 (mt fusA1 fusB2). As expected when this cross was performed two types of plasmodium 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 CLd (fusA2 fusB1). 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 germinated. A 1:1 ratio of mt₄:mt₁ was, as expected, observed in these clones. All homothallic clones were of the delayed type. From amongst these mt₄ clones one of type fusA₂ fusB₁ was selected and designated OU₃h3.

Table 1. Analysis of progeny clones from the cross LU₆₄₈ × i (mt₁ fusA₁ fusB₁ × mt₂ fusA₂ fusB₂)

| Genotype of clone | LU₆₄₈ | LU₆₈₈ | i | No. of clones |
|-------------------|--------|--------|---|--------------|
| mt₁ fusA₁ fusB₁   | No plasmodia | I | IV | 7 |
| fusA₁ fusB₂       | II     | IV    | 9 |
| fusA₂ fusB₁       | III    | VI    | 4 |
| fusA₂ fusB₂       | IV     | VI    | 12 |
|                   |        |       | 32 |
| mt₂ fusA₁ fusB₁   | I       | No plasmodia | No plasmodia | 6 |
| fusA₁ fusB₂       | II     | 11    |
| fusA₂ fusB₁       | III    | 6     |
| fusA₂ fusB₂       | IV     | 6     |
|                   |        | 29    |

(ii) Production of a mixed plasmodium derived from clones of genotype mt₄ (delayed) fusA₁ fusB₂ and mt₄ (delayed) fusA₂ fusB₁

Plasmodia of this type should be formed when clones OU₃h3 and CLd 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 (fusA₁/fusA₂, fusB₁/fusB₂) and the selfed plasmodia of fusion groups II (fusA₁ fusB₂) or V (fusA₂ fusB₁). OU₃h3 and CLd 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 OU₃h3), 28 were of fusion group V (selfed CLd), and one was of mixed type (fusion group IV).
(iii) **Analysis of progeny clones from the mixed plasmodium OUh3 x CLd**

The plasmodium **OUh3 x CLd** was sporulated and 183 amoebal clones isolated (Table 2). All these clones produced plasmodia; confirming that both of the parental types were **mt**. One hundred and ten of these clones formed plasmodia rapidly in plaques (resembling **CL**) while 73 formed plasmodia rarely and after a delay (resembling **OUh3** and **CLd**). 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 OUh3 x CLd (mt fusA fusB x mt fusA fusB)**

| Genotype of clone | Fusion class of selfed clone | Plasmodial formation | No. of clones | Totals |
|-------------------|----------------------------|----------------------|---------------|-------|
| mt fusA fusB      | I                           | Delayed              | 11            | 38    |
|                   |                             | Rapid                | 27            |       |
| mt fusA fusB      | II                          | Delayed              | 16            | 43    |
|                   |                             | Rapid                | 27            |       |
| mt fusA fusB      | V                           | Delayed              | 21            | 44    |
|                   |                             | Rapid                | 23            |       |
| mt fusA fusB      | 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 **fusA**). 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 **OUh3 x CLd** was diploid, and that recombination had occurred between the unlinked loci **fusA** and **fusB** 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 **OUh3**. A majority of the group V progeny plasmodia gave killing when fused with a plasmodium derived from parental clone **CLd**. 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<sub>h</sub> amoebal strains. In order to do this a plasmodium was produced by mixing two mt<sub>h</sub> (delayed) clones carrying appropriate genetic markers. The progeny cloned from spores of such a plasmodium show free recombination between the genetic markers *fusA* and *fusB*. Therefore the nuclei from the two original amoebal clones must have fused. There is therefore no possibility that the mixed plasmodium *OUh3 × CLd* 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 *OUh3 × CLd* was a doubly heterozygous diploid. In order to detect recombination more than one locus must be heterozygous, and since *mt<sub>h</sub>* must be homozygous in this analysis the most suitable choice of genetic markers was *fusA* and *fusB*.

If, using these markers, diploid plasmodia can be found derived from *mt<sub>h</sub>* clones, then it is also probable that within a pure clone of *mt<sub>h</sub>* 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<sub>h</sub>* 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<sub>h</sub> 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 *OUh3 × CLd* requires some explanation (both *OUh3* and *CLd* were delayed homothals). The ratio of rapid to delayed *mt<sub>h</sub>* alleles in the *OUh3 × CLd* progeny clones approximates 1:1. Cooke & Dee (1975) summarized some information relevant to this problem. They reported that when *CLd* was crossed with heterothallic clones and the (diploid) plasmodium sporulated the progeny clones showed *mt<sub>h</sub>* (delayed) or heterothallic characters, and that these occurred in the ratio 1:1. This observation, which is confirmed by the present analysis of *OUh3 × CLd*, suggests that the *mt<sub>h</sub>* (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 $mth_{(\text{delayed})}$ character is a mutant form of the $mth_{(\text{rapid})}$ character. This mutant form is stable through the meiotic cycle. However, during growth of a clone of the $mth_{(\text{delayed})}$ 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 $mth_{(\text{delayed})}$ cannot give rise to plasmodia unless it has reverted to $mth_{(\text{rapid})}$. This hypothesis would explain the appearance of the rapid homothallic character in the present analysis.

A mixed culture of clones $OUh3$ ($mth_{\text{delayed}}$) and $CLd$ ($mth_{\text{delayed}}$) would be unable to give rise to the diploid plasmodium $OUh3 \times CLd$ unless one or other of the clones reverted to the $mth_{(\text{rapid})}$ type. Following such a reversion of an amoeba of one clone or the other the resulting mixed plasmodium would be heterozygous for the characters $mth_{(\text{rapid})}$ and $mth_{(\text{delayed})}$. The progeny clones would, as reported, show a 1:1 segregation for this character.

If this is correct then $OUh3 \times CLd$ can be viewed as not simply a double heterozygote (for $\text{fusA1}/\text{fusA2}$, and $\text{fusB1}/\text{fusB2}$) but a triple heterozygote involving also $mth_{(\text{rapid})}$. This hypothesis suggests that the $mth_{(\text{rapid})}$ character is dominant to the $mth_{(\text{delayed})}$ character. In the cross $mth_{(\text{delayed})} \times$ 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 $mth$ 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 $mth_{(\text{delayed})}$ character is an allelic form of the $mth_{(\text{rapid})}$ 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 $mth_{(\text{rapid})}$ and $mth_{(\text{delayed})}$ 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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