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Selfing mutants from heterothallic strains of *Physarum polycephalum*

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SUMMARY

Plasmodial formation in the myxomycete *Physarum polycephalum* is controlled by a mating type (*mt*) locus, with heterothallic amoebae normally being unable to form plasmodia in pure clones. We report the isolation by mutagenesis of selfing mutants from heterothallic strains, and their analysis. Various amoebal strains of different mating types were mutagenized with a range of mutagens, and a number of selfing mutants (designated Het$^-$) were isolated. A specific sensitivity of mt_2 amoebae to mutagenesis by NMG was observed. This sensitivity segregated as a single locus closely linked or allelic to the mt_2 locus. When the Het$^-$ clones were incubated at 30 °C, selfing was greatly inhibited. This property was used to determine the *mt* specificities of four Het$^-$ clones. The process of plasmodial induction in pure clones of *CL* was also studied using the 30 °C temperature effect.

1. INTRODUCTION

The myxomycete *Physarum polycephalum* is a simple eukaryote with a readily observed differentiation step that is amenable to genetic analysis. In this differentiation microscopic, uninucleate amoebae develop into macroscopic, syncytial, multinucleate plasmodia (Gray & Alexopoulos, 1968). Diploidization normally accompanies this process. The development of amoebae into plasmodia has aroused considerable interest and much work has been done on the system (e.g. Anderson & Dee, 1977; Davidow & Holt, 1977; Wheals, 1973).

Plasmodial formation in *P. polycephalum* is controlled by a large number of alleles at the mating type (*mt*) locus. Heterothallic amoebae are only able to form plasmodia when amoebae with different *mt* alleles are combined (Dee, 1973). Amoebae derived from the Colonia isolate possess the allele mt_h and are able to form plasmodia in pure clones (i.e. 'self') (Wheals, 1970). The selfed plasmodia derived from Colonia are haploid, unlike the crossed plasmodia derived from heterothallic strains (Anderson, Cooke & Dee, 1976; Cooke & Dee, 1974; Mohberg, 1977). A number of mutants have been isolated from Colonia that are unable to

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self (Anderson & Dee, 1977; Davidow & Holt, 1977; Honey, Poulter & Teale, 1979; Wheals, 1973).

Heterothallic amoebae normally do not self, but Adler & Holt (1975) report a number of heterothallic strains that self at a low frequency, these being derived from hybrids from crosses between Wisconsin and Indiana strains. The observation that selfing clones might be derived from heterothallic clones is significant, and further investigation of the subject is desirable. We report the isolation of a number of such selfing clones by mutagenesis of heterothallic strains, and their analysis. As these mutants have lost their heterothallic controls over plasmodial formation, they are designated as Het$^-$. A preliminary report of this work has been made in abstract (Poulter, Honey & Teale, 1977), and similar results have also been reported by Adler & Holt (1977) and Gorman, Dove & Shaibe (1979).

If a culture of *CL* amoebae is incubated at 30 °C, the formation of plasmodia is greatly inhibited (Adler & Holt, 1974). We report a study of the process of plasmodial induction in *CL* using the effect of elevated temperatures.

2. MATERIALS AND METHODS

(i) *Strains LU648*: mt_1, $fusA_1$, $fusB_1$. *LU688*: mt_2, $fusA_1$, $fusB_1$. *a*: mt_1, $fusA_1$, $fusB_1$. *i*: mt_2, $fusA_2$, $fusB_2$. *LU863*: mt_4, $fusA_1$, $fusB_1$. *LU913*: mt_3, $fusA_1$, $fusB_1$. *CL*: mt_h, $fusA_2$, $fusB_1$. *OUD1*: mt_2, $fusA_1$, $fusB_2$. *OUD3*: mt_1, $fusA_2$, $fusB_2$. *OUD7*: mt_1, $fusA_1$, $fusB_2$. The origins of these strains have been described previously (Anderson & Dee, 1977; Cooke & Dee, 1975; Dee, 1966; Poulter & Honey, 1977).

(ii) *Loci mt, mating type.* Heterothallic alleles mt_1, mt_2, mt_3, mt_4 (Dee, 1966) and selfing allele mt_h (Wheals, 1970). *fusA*, *fusB*, plasmodial fusion type, with alleles $fusA_1$, $fusA_2$, $fusB_1$, $fusB_2$ (Dee, 1973).

(iii) *Experimental methods.* Amoebae and plasmodia were cultured on semi-defined medium (SDM) as described by Honey *et al.* (1979). The experimental techniques used in this work have been described previously (Honey, 1979). Heterothallic amoebae were mutagenized with *N*-methyl *N'*-nitro *N*-nitroso-guanidine (NMG) by adding 0·05 ml of a 100 μg/ml solution to the centre of each amoebal plate, similar to the mutagenesis of *CL*. At each point of application a zone about 1·0 cm in diameter occurred in which all amoebae and bacteria were killed. Assuming the NMG diffused evenly throughout the 25 ml agar in each plate, the NMG reached a final concentration of 200 ng/ml. Following mutagenesis selfing mutants appeared evenly throughout each plate, except that none formed within the central zone.

A similar procedure was employed in the mutagenesis of heterothallic strains using the mutagens ethyl methane sulphonate (EMS), acriflavine, aflatoxin, beno(a)pyrene, and ethionine. Heterothallic amoebae were also mutagenized using UV-irradiation. The amoebal strains were subcultured on to a number of SDM plates and exposed to a Philips 57413, P/40 TUV strip lamp such that there was an approximately 50% survival frequency. The plates were then spread with *Escherichia coli* suspension and incubated at 26 °C.

3. RESULTS

(i) *Mutagenesis of heterothallic strains*

No heterothallic amoebal strains had been observed to form plasmodia in pure clones in our laboratory under routine culture conditions. Adler & Holt (1975) reported, however, that some heterothallic strains do self on a rare basis, with their data suggesting that some of these selfing strains were aneuploid. The selfing phenotypes of a range of heterothallic strains were therefore determined before mutagenesis of them was undertaken. Ten plates of each of the strains *LU648*, *LU688*, *a*, *i*, *OUD1*, *OUD3*, and *OUD7* were subcultured and incubated at 26 °C for 30 days. The plates were checked every day for the appearance of plasmodia.

A small number of plasmodia formed on three of the plates of *OUD3* after 15–21 days' incubation. One of these plasmodia was isolated and found to be fusion class VI (i.e. $fusA_2, fusB_2$). The plasmodium was sporulated and the spores germinated. The spores had a 0·1 % viability and the progeny clones retained the parental character; they only selfed on a very rare basis in large amoebal plaques. No plasmodium was observed on any culture of any of the other amoebal strains tested, and they were therefore suitable for mutagenesis.

Ten plates of *LU688* were mutagenized with NMG as described above and a number of plasmodia became visible between six and 22 days, with a majority appearing between six and ten days. These plasmodia were considered to represent selfing mutants, defective in some gene involved in heterothallism, and were designated Het$^-$. The total number of Het$^-$ mutants formed on any particular plate could be counted if each newly formed plasmodium was counted and destroyed with a hot needle as soon as it was detected.

A further 80 plates of *LU688* were mutagenized using a range of initial concentrations of NMG from 0·1 μg/ml to 200 μg/ml (final concentrations from 0·2 ng/ml to 400 ng/ml). Increased numbers of plasmodia were observed with increased NMG concentrations, from 0 per plate at 0·1 μg/ml NMG to 33 plasmodia per plate at 200 μg/ml NMG. A range of amoebal numbers (from 60 to 500 per plate) were also subcultured and 100 μg/ml NMG added after 3–4 days' incubation. The increasing densities of amoebae on the plates did not greatly affect the number of mutants formed.

(ii) *Effects of different mutagens and* mt *alleles*

Six amoebal strains of four different mating types were mutagenized with 100 μg/ml NMG and UV (Table 1). In addition, duplicate plates of *LU648* and *LU688* were mutagenized with a range of concentrations of each of the mutagens EMS (four volumes between 1 and 8 μl added), aflatoxin, acriflavine, benzo(a)-pyrene, and ethionine (each with six initial concentrations between 0·5 and 12 mg/ml). A single Het$^-$ mutant formed on a plate of *LU688* mutagenized with 2 μl EMS but it died before it could be subcultured. No plasmodium was observed on any other plate mutagenized with these five mutagens. The number of Het$^-$ mutants formed following NMG mutagenesis varied over a wide range, depending on the mating type of the strain mutagenized. UV mutagenesis induced a steady, low number of Het$^-$ mutants.

The difference in the number of plasmodia formed after mutagenesis of mt_1 and mt_2 strains by NMG was studied further. The cross *LU648* × *LU688* was made and the plasmodium sporulated. Eighty-four amoebal progeny clones were isolated and each crossed with *LU648* and *LU688* in order to determine its mating types. Each progeny clone was also mutagenized with NMG and the number of plasmodia formed counted (Table 2). The differing response to NMG was still present in the

Table 1. *Mutagenesis of heterothallic strains. Average numbers of Het⁻ mutants formed per plate*

Mating-type strain	mt_1		mt_2		mt_3	mt_4
	LU648	*a*	*LU688*	*i*	*LU913*	*LU863*
NMG						
No. of plates	45	8	39	14	10	10
No. of plasmodia	6	2	1144	118	42	3
Plasmodia per plate	0·13	0·25	29	8·4	4·2	0·3
UV						
No. of plates	96	—	33	—	10	10
No. of plasmodia	5	—	1	—	1	1
Plasmodia per plate	0·05	—	0·03	—	0·1	0·1

Table 2. *NMG mutagenesis of progeny clones from the cross LU648 × i*

(One plate of each progeny clone was mutagenized and the number of plasmodia formed counted. The number of progeny clones each forming a particular number of plasmodia were counted)

No. of plasmodia formed by each progeny clone	No. of progeny clones	
	mt_1	mt_2
0	39	0
1–10	6	2
11–20	0	9
21–30	0	10
31–40	0	11
41–50	0	5
51–60	0	1
61–70	0	1

progeny and segregated as a single locus with the *mt* locus. One mt_2 progeny clone gave rise to a single plasmodium after mutagenesis, and the rest gave rise to eight or more. Six mt_1 progeny clones each gave rise to a single plasmodium.

(iii) *Progeny of Het⁻ mutants*

The original Het⁻ plasmodia were isolated from plates of heterothallic amoebae, and thus the selfing phenotypes of the Het⁻ mutant amoebae from which they were derived were not known. That is, it was not known whether the mutant Het⁻ amoebae selfed rapidly or rarely to form the resulting Het⁻ plasmodia. A number

of Het⁻ mutants were, therefore, investigated further. Thirteen of the Het⁻ plasmodia derived from *LU688* were fusion tested and, as expected, all were fusion class I (i.e. $fusA_1 fusB_1$). Five hundred and sixty-seven Het⁻ plasmodia were sporulated and the spores germinated.

The amoebal progeny clones were scored for their selfing phenotypes (Table 3). Seventy-nine of the clones selfed rapidly in small plaques (similar to *CL*). Three hundred and ninety-three clones either selfed on a rare basis (similar to *CLd*) or did not self at all after 14 days' incubation. Seven of these rare-selfing clones were analysed further and found to retain the *mt* specificity of their parental heterothallic strains. Ninety-five of the Het⁻ mutants did not have viable spores.

Table 3. *Selfing phenotypes of progeny clones of Het⁻ plasmodia. Number of progeny clones of each type*

Parental strain	Mutagen	Progeny phenotype: Rapid selfing	Rare selfing	Spores not viable
LU688	NMG	77	378	92
	UV	0	2	0
LU648	NMG	2	8	3
	UV	0	5	0

(iv) *Plasmodial induction*

If amoebal clones of *CL* are incubated at 30 °C, the formation of selfed plasmodia is greatly inhibited (Adler & Holt, 1974). A number of rapid-selfing Het⁻ mutants were incubated and a similar effect observed. This inhibition of selfing at 30 °C was used to study the process of plasmodial induction in selfing strains. We consider *CL* to be a selfing strain of the same type as the Het⁻ mutants, and much of this work involved the use of *CL*.

When *CL* was incubated at 30 °C, we found that no selfing occurred for at least five days. After this period rare selfing did occur, becoming frequent after seven days' incubation. The effect was very sensitive to slight variations in temperature. Above 31 °C, amoebal growth was very poor and no plasmodia grew. Between 30 and 31 °C there was good amoebal growth with little plasmodial formation. At temperatures less than 30 °C selfing was not inhibited, many plasmodia becoming apparent after 60 h incubation. Any *CL* plasmodia that formed at 30 °C, or that were transferred from 26 °C, grew very readily at the higher temperature. The effect of elevated temperature on *CL*, therefore, was to inhibit the formation of plasmodia and not to prevent the growth of plasmodia already formed.

The time course of plasmodial induction was studied. Twenty-six plates of *CL* were incubated at 26 °C for varying periods of time before placing pairs of plates at 30 °C. The time when plasmodia first became visible under the binocular microscope was noted for each plate (Fig. 1). If *CL* was incubated for less than 34 h at

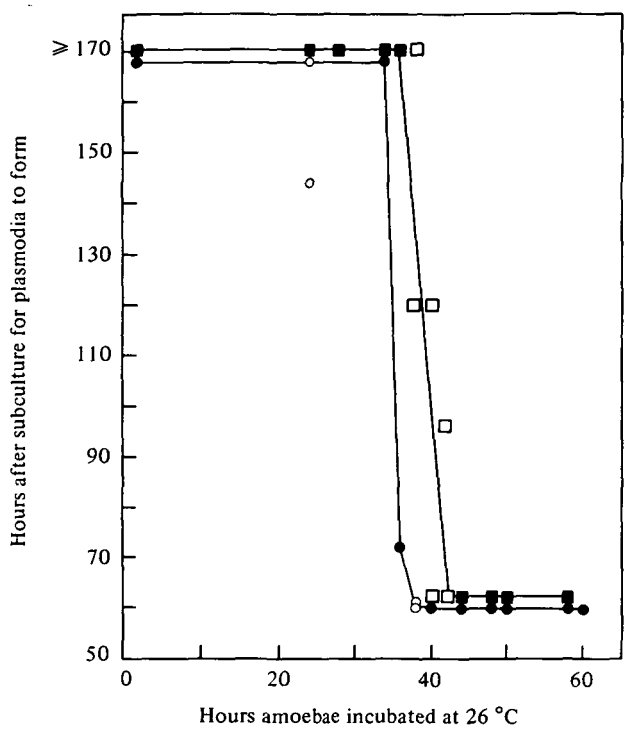

Fig. 1. Plasmodial induction in *CL* and *NH45*. Effects of incubation at 26 °C before placing at 30 °C. ○, Single plate of *CL*. ●, Duplicate plates of *CL*. □, Single plate of *NH45*. ■, Duplicate plates of *NH45*.

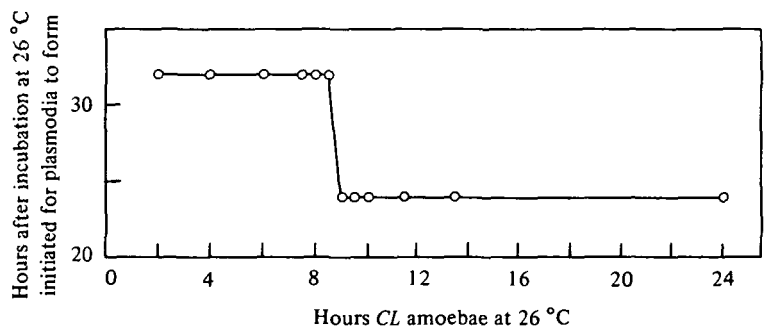

Fig. 2. Plasmodial induction in *CL*. Effects on plaques previously grown at 30 °C of incubation at 26 °C before returning to the higher temperature.

26 °C before being placed at 30 °C, plasmodial formation remained fully inhibited. If *CL* was incubated at 26 °C for longer than 36 h, there was no inhibition of selfing. There is evidently a temperature-sensitive step that ends after 36 h incubation. A similar experiment was performed with the Het⁻ mutant, *NH45* (derived from *LU648* by NMG mutagenesis), and a similar result obtained (Fig. 1). The temperature-sensitive step was finished after about 40 h incubation. The

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duration of the temperature-sensitive step was then studied. Twenty-four plates of *CL* were incubated at 30 °C for 5 days, then placed at 26 °C. After varying periods of time, pairs of plates were returned to 30 °C and the appearance of plasmodia carefully noted (Fig. 2). If *CL* was incubated at 26 °C for longer than 9 h, plasmodial formation became irreversible. The temperature-sensitive step is of 9 h duration.

Table 4. *Crosses between Het⁻ clones and heterothallic strains at 30 °C. Number of days for crossed plasmodia to form*

		Het⁻ derived from *LU688* (mt_2)			Het⁻ derived from *LU648* (mt_1)
		NH34	*NH36*	*NH48*	*NH45*
mt_1	*OUD3*	4	4	3	—
	OUD7	4	3	3	—
mt_2	*OUD1*	6	—	—	5
	i	6	—	—	2

—, No crossed plasmodia formed.

(v) *Het⁻ crosses at 30 °C*

Although the Het⁻ mutants which self rapidly at 26 °C do not self readily at 30 °C, they do cross with heterothallic strains at this temperature (Adler & Holt, 1977). This property was used to determine the *mt* specificities of four of the rapid-selfing Het⁻ mutants isolated in this work. The mutants were crossed with a range of heterothallic strains at 30 °C, and the resulting plasmodia fusion tested (Table 4). Each Het⁻ mutant retained the *mt* specificity of its original heterothallic strain, except for *NH34* which had partially lost its mt_2 specificity and exhibited a crossing pattern similar to *CL*.

4. DISCUSSION

We report that rapidly selfing mutant clones can be isolated by mutagenesis of heterothallic amoebal strains. The genetic properties of *CL* and the Het⁻ mutants are very similar, and we suggest that *CL* is a selfing mutant of the same type. It seems likely that, as for *CL*, plasmodial formation in the Het⁻ mutants occurs by an apogamic mechanism.

Of the four mutants characterized further, *NH34* had undergone a partial loss of its mt_2 specificity, resembling the specificity of *CL*, whereas *NH36*, *NH48*, and *NH45* all retained their *mt* specificities. We will present evidence in a future report indicating that the Het⁻ mutations are closely linked or allelic to the *mt* locus. The progeny of only a minority of the Het⁻ mutants had the rapid-selfing phenotypes, whereas the progeny of the majority only selfed rarely or apparently not at all. Adler & Holt (1977) and Gorman *et al.* (1979) report similar results with other selfing mutants. Most of the original Het⁻ mutations may not have resulted in a rapid selfing phenotype. Some may have been induced by a 'leaky' mutation, and

it is possible that *D3* is a naturally occurring variant that is similarly leaky. *NMG* was more effective in producing Het⁻ mutations in mt_2 amoebae than in amoebae of other mating types. There seems to be a close relationship between the different *mt* alleles and the mode of mutagenic action of *NMG*. The other mutagens tested all had a low effectiveness in the induction of Het⁻ mutations.

The isolation of the selfing mutants indicates that all the genetic information required for plasmodial formation is present within each heterothallic clone. Plasmodial formation in *CL* occurs by an apogamic mechanism, showing that neither cell fusion nor nuclear fusion events are necessary to initiate this process. The nature of the Het⁻ mutation is uncertain, but it presumably unmasks a function that induces plasmodial formation. This unknown function would normally be unmasked in normal mating by the interaction of two heterothallic amoebae.

The studies of the plasmodial induction of *CL* suggest the following scheme of events for incubation at 26 °C. Following subculture of *CL*, there is an initial growth period of 27 h which is not affected by incubation at 30 °C. This may be a growth phase in which the amoebae must deplete the available food supply and enter a state of starvation before they can become competent to initiate plasmodial formation. Youngman *et al.* (1977) found that food depletion is an important event in plasmodial formation.

After this initial period, a temperature-sensitive process necessary for plasmodial formation is initiated which lasts for 9 h. After the 9 h, plasmodial induction is once more insensitive to heat. Plasmodia become visible under the binocular microscope 60 h after subculture. The plasmodia form in the region where there is active amoebal growth, and it is interesting to speculate whether it is necessary for the amoebae to undergo a complete mitotic cycle (about 8–9 h duration) at the permissive temperature before they are committed to plasmodial formation. It is likely that this mode of plasmodial induction is common for all the Het⁻ mutants.

Although Het⁻ amoebae do not self readily at 30 °C, they will cross with heterothallic strains. Also, two heterothallic strains will cross readily at the elevated temperature. The reason for the difference is uncertain but may be related to the different haploid and diploid modes of plasmodial development. It would be of interest to determine by measuring DNA contents whether the rare selfed *CL* plasmodia obtained at 30 °C are diploid.

In conclusion, selfing mutants can be isolated from heterothallic amoebae. The further analysis of these mutants should provide valuable insight into the genetic control of plasmodial formation in *P. polycephalum*.

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