

Methods for the isolation and analysis of plasmodial mutants in *Physarum polycephalum*

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(Received 3 June 1974)

SUMMARY

Amoebae of the Colonia isolate of *Physarum polycephalum* produce plasmodia within individual amoebal clones.

This paper reports the derivation from the Colonia strain C50 of a strain CL (Colonia Leicester) which produces plasmodia in clones with high efficiency and which completes the life cycle reliably and repeatedly in single clones. The derivation of a line CLd (CL delayed plasmodium formation) is described and, using CLd, the construction of the isogenic heterothallic strains $mt_1;CL$ and $mt_2;CL$.

The above strains provide a system for the isolation and genetic analysis of mutants of *P. polycephalum* within a uniform genetic background, in particular mutants expressed in the plasmodium.

Using this system two auxotrophic mutants have been isolated. Preliminary genetic analysis has shown that they are due to single gene mutations.

I. INTRODUCTION

The value of the Myxomycete *Physarum polycephalum* as an organism for the study of developmental processes, general cellular properties and in particular the cell cycle has been discussed elsewhere (Hutterman, 1973). Due to the lack until now of suitable genetic techniques, biochemical studies of *P. polycephalum* in these fields have not yet been complemented by equivalent genetic studies. The recent work by Wheals (1970, 1973), Haugli & Dove (1972) and Dee, Wheals & Holt (1973) on mutagenesis and genetic analysis in *P. polycephalum* has however provided a firm basis for the advancement of genetic studies in this organism. The rapid further development of techniques for genetic analysis in *P. polycephalum* is therefore of great importance.

Prior to 1970, only heterothallic strains, in which haploid amoebae of differing mating type fused to form a diploid plasmodium, had been described. Thus to produce plasmodia homozygous for a particular mutation, after amoebal mutagenesis and selection, a tedious series of outcrosses and backcrosses was required. This difficulty was circumvented by the work of Wheals (1970) who showed that amoebae of the Colonia isolate of *P. polycephalum* produced plasmodia within individual 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 due to a single allele at the mating type (*mt*) locus which he designated *mt_h*. Measurements of nuclear DNA content of *mt_h* amoebae and clonally produced plasmodia (Cooke & Dee, 1974) have shown that there is no change in ploidy during plasmodium formation by *mt_h* amoebae, the plasmodia presumably remaining haploid. The advantage of using *mt_h* amoebae for mutant isolation should be immediately apparent: mutations detected in the amoebal stage can be easily tested for in the plasmodial stage since a plasmodium can be produced directly from a single amoebal clone. Similarly recessive mutants expressed only in the plasmodial stage can be isolated by screening plasmodia derived from single amoebal clones.

After amoebal mutagenesis, it is necessary to screen large numbers of clonally derived plasmodia in order to isolate plasmodially expressed mutants. Thus mutagenized *mt_h* amoebae must be plated on a bacterial lawn so that they form well-separated colonies or plaques. In the absence of specific enrichment techniques plasmodia derived from such plaques have to be screened individually. However *C50* amoebae would not normally produce plasmodia in plaques, but only when grown on a dense bacterial streak. Wheals (1973) isolated a strain *C5-1*, derived from *C50* after mutagenesis which produced plasmodia in plaques with 100% efficiency; however, *C5-1* plasmodia failed to sporulate.

The first aim of the present work, therefore, was to derive an *mt_h* strain of *P. polycephalum* which, unlike previously reported strains, would produce plasmodia in plaques with 100% efficiency and in which all plasmodia would be capable of completing the life-cycle.

In order to analyse genetically mutants isolated in an *mt_h* strain the construction of heterozygous diploids is essential. Though present evidence suggests that no nuclear fusion occurs when mixtures of *mt_h* amoebae form plasmodia (Cooke & Dee, 1974), it has been demonstrated that *mt_h* and heterothallic amoebae can cross to form heterozygous diploid plasmodia (Wheals, 1970). However, difficulties would arise if crosses were made between *mt_h* mutant amoebae and amoebae of distantly related heterothallic isolates, since the strains may differ grossly in their chromosome complement (Mohberg *et al.* 1973). The results of such crosses might be impossible to interrupt. The second aim of the present work therefore was to construct strains of heterothallic mating types which were as far as possible isogenic with the strain isolated for mutant selection.

2. MATERIALS AND METHODS

(i) *Strains*. The heterothallic amoebal strains *a* and *i* have been previously described (Dee, 1966). Strain *LU1* is a progeny clone of the plasmodium $a \times ai:36$ ($ai:36$ is a progeny clone of an $a \times i$ plasmodium). The isolation of the Colonia strain *C50* was described by Wheals (1970).

(ii) *Loci*. *mt*, mating type. Alleles *mt₁*, *mt₂*, heterothallism (Dee, 1966); *mt_h*, plasmodium formation within amoebal clones (Wheals, 1970; Cooke & Dee, 1974).

fusA and *fusB*, plasmodial fusion type. Identity at both *fusA* and *fusB* loci is

a prerequisite for plasmodial fusion; *fusB2* is dominant to *fusB1*; *fusA1* and *fusA2* are co-dominant.

The loci *fusA* and *fusB* controlling plasmodial fusion were previously designated *f* and *n* respectively (Poulter & Dee, 1968; Poulter, 1969). The change was made to conform with currently accepted principles of nomenclature and has been agreed with workers in several other laboratories.

sax, sensitivity to axenic medium (Poulter, 1969; Wheals, 1973). Plasmodia homozygous for *sax*⁻ fail to grow when subcultured to axenic medium (SDM).

(iii) *Genotypes of amoebal strains*. *a:mt₁ fusA1 fusB1 sax*⁻. *LU1:mt₂ fusA1 fusB1 sax*⁻. *C50, CL, CLd:mt_h fusA2 fusB1 sax*⁺.

(iv) *Cultural conditions*. Amoebae were maintained in two-membered culture with *Escherichia coli* at 26 °C on liver infusion agar, LIA (1 g Oxoid liver infusion powder/1 l. 2 % agar). All plasmodia were routinely cultured at 26 % on a semi-defined medium, SDM (Dee & Poulter, 1970). Production of spores, spore plating and isolation of progeny clones were carried out as previously described (Wheals, 1970).

(v) *Plasmodium formation*. Plasmodium formation by amoebae derived from a single clone was achieved by inoculating a dilute SDM agar plate (6.5 ml liquid SDM/100 ml 2 % agar) with approximately 10³ amoebae together with a drop of bacterial suspension, allowing the drop to absorb and incubating for 3–5 days. The same procedure was adopted for crossing; the drop of bacterial suspension being inoculated with amoebae of different mating types. The resulting plasmodia were subcultured to SDM agar containing 250 µg/ml streptomycin (SDMS) to kill any remaining bacteria.

(vi) *Plasmodial fusion tests*. Methods have been fully described by Poulter & Dee (1968).

(vii) *Amoebal mutagenesis*. The methods used were adaptations of those described by Haugli, Dove & Jimenez (1972), Haugli & Dove (1972) and Wheals (1973). A 60 mm Oxoid membrane filter (pore size 0.45 µ) was inoculated and spread with 0.1 ml of amoebal suspension (5 × 10² cells/ml) together with 0.1 ml of a bacterial suspension. The membrane filter, resting on filter paper, was supported by a layer of glass beads in a covered Petri dish and was underlaid with 20 ml liver-infusion solution. After 5 days' incubation, when the cell density had reached approximately 2 × 10⁵ cells per filter the amoebae were exposed to either ultra-violet (UV) light or the chemical mutagen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NMG).

For UV mutagenesis the cells were irradiated at a dose of 800 ergs/mm² (dose rate 25 ergs/mm²/s) giving about 4 % survival after a 24 h period of post-irradiation growth to allow for segregation of mutants. Caffeine (0.025 %) was added to the growth medium to inhibit DNA repair enzymes.

For NMG mutagenesis the membrane filters were transferred to dishes containing 250 µg/ml NMG in phosphate buffer (pH 7.0) for 30 min and then transferred to 2 % sodium thiosulphate solution (10 min). Two further transfers were made to fresh LIA solution (10 min each) after which the filters were returned to their

original dishes and incubated for a further 24 h. The procedure gave a survival rate of 30–50 %.

For selection of temperature-sensitive mutants pre- and post-mutagenic incubation was at 23 °C rather than 26 °C.

(viii) *Plasmodial test media*. DM-1, minimal defined medium (Dee *et al.* 1973). DM-3, DM-1 plus 50 µg/ml alanine, valine, proline, tryptophan, serine, threonine, cysteine, arginine, histidine, lysine, aspartic acid, leucine and isoleucine.

(ix) *Progeny analysis*. To determine the mating type of progeny clones from a cross, amoebae taken from randomly selected plaques on spore germination plates were subcultured, recloned and tested for their ability to form plasmodia. The amoebae were inoculated densely into a drop of bacterial suspension, alone and in combination with tester amoebae of different heterothallic mating type. Progeny clones which gave plasmodia only when mated with tester amoebae were classified for their heterothallic mating type. Those which gave plasmodia when inoculated alone (and with all tester amoebae) were classified as mt_h . The tester strains for the mating type tests were of appropriate genotype to allow the classification of resultant plasmodia for *sax* and *fus* alleles. This permitted the determination of the genotype of the heterothallic progeny clones. Plasmodia which failed to grow when subcultured to SDM agar were classified as *sax*⁻. Viable plasmodia were tested for their fusion behaviour against appropriate testers.

3. RESULTS

(i) *Isolation of strain CL (Colonia Leicester)*

It was observed by Dee and Holt that spores obtained from a *C50* plasmodium, when germinated on DSDM gave rise to plaques of amoebae all of which developed plasmodia, in contrast to *C50* amoebae which would not form plasmodia in plaques. *C50* spores were therefore chosen as the starting material for derivation of an mt_h clone which would form plasmodia in clones with high efficiency and would complete the life-cycle within clones with no apparent heterogeneity. Spores from a *C50* plasmodium were germinated on a bacterial lawn on LIA plates. Nine progeny amoebal plaques, *C50*-102 to *C50*-110, were isolated (Fig. 1). Amoebae from each were then plated at a density of approximately 50 per plate together with a bacterial suspension on DSDM agar to test their ability to form plasmodia within single amoebal plaques (Fig. 1). Also four of the amoebal cultures *C50*-103, 105, 107 and 109, were recloned and four clones of each were tested for their ability to form plasmodia in plaques (Fig. 1). All lines produced plasmodia in all amoebal plaques. Incubation was continued until plasmodia derived from individual plaques on a single test plate had fused into a single large plasmodium. Each plasmodium carried the designation of the amoebal line from which it was derived. The plasmodia were then tested for their ability to produce spores. All plasmodia derived from progeny clone *C50*-105 failed to sporulate (Fig. 1). A degree of heterogeneity is thus indicated in the original *C50* plasmodium. The spore batches were germinated and plated on DSDM. All remaining lines produced plasmodia in

all amoebal plaques. Spores of one clonally derived plasmodium, C50-1094, were tested for their ability to produce progeny clones capable of repeating the life-cycle. Progeny amoebae of C50-1094 were recloned and four clones were tested for formation of plasmodia in plaques. In all cases plasmodia were produced in 100% of amoebal plaques. These plasmodia under appropriate conditions produced spores whose progeny were again able to repeat the life-cycle.

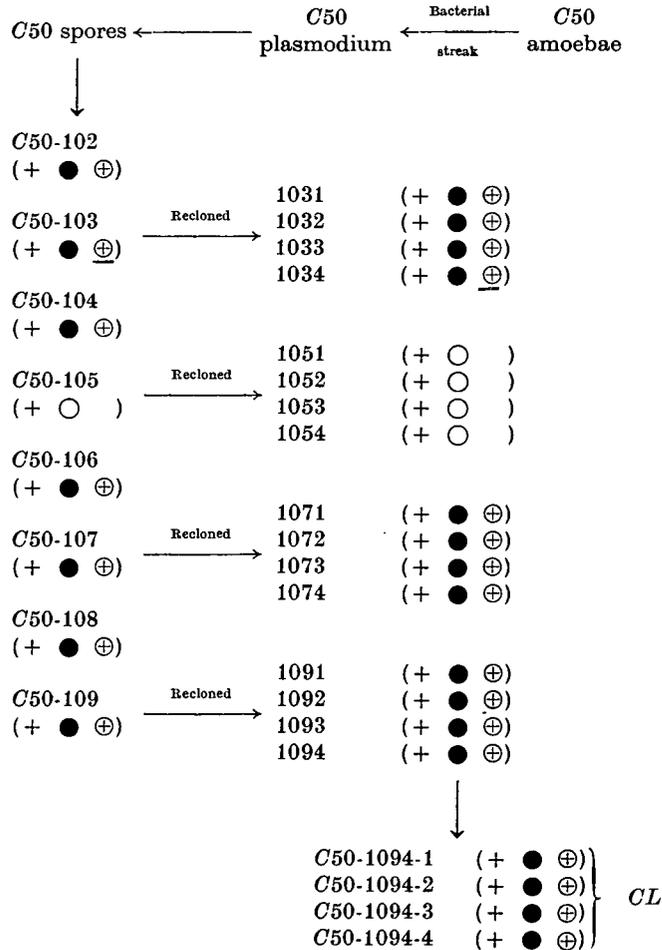


Fig. 1. Derivation of strain CL. + when tested all observed amoebal plaques produced plasmodia. At least 50 plaques were scored in each case. ● Plasmodia produced spores under appropriate conditions. ○ Plasmodia failed to produce spores under appropriate conditions. ⊕ Subsequent generation; when tested all observed amoebal plaques produced plasmodia.

Thus the plasmodium C50-1094, itself derived from an individual amoebal clone, fulfilled the criterion of producing progeny capable of repeating the life-cycle within individual clones with no apparent heterogeneity. For this reason its progeny were selected to form a standard strain for future work and designated CL

(Fig. 1). *CL* amoebae may be maintained without plasmodia formation by regular subculturing (every 3–4 days) or by storage at 4 °C. *CL*, like *C50* and *C5-1*, was shown by plasmodial fusion tests to have the genotype *fusA2 fusB1*.

(ii) *Crosses between CL and heterothallic strains*

In order to construct heterothallic strains isogenic with *CL* it was necessary to cross *CL* (mt_h) amoebae with amoebae of heterothallic strains. However when such a cross is attempted, two types of plasmodia may arise, namely those derived directly from mt_h amoebae and those resulting from crossing between heterothallic and mt_h amoebae. If heterothallic strains of suitable genotype are used it is possible to distinguish the two classes of plasmodia by means of the plasmodial fusion system (Poulter & Dee, 1968; Wheals, 1970). Thus a plasmodium which resulted from a cross between amoebae of the genotype $mt_1 fusA1 fusB1$ (*a*) and $mt_h fusA2 fusB1$ (*CL*) would have the hybrid genotype *fusA1/fusA2, fusB1/fusB1* (fusion group III) whereas plasmodia formed in clones of *CL* amoebae fuse with tester plasmodia of fusion group V (*fusA2/fusA2, fusB1/fusB1*). Therefore of the plasmodia forming on a cross plate, those arising directly from mt_h amoebae might be expected to behave as group V plasmodia and those arising from a cross as group III plasmodia.

Initially the standard procedure for setting up heterothallic crosses was adopted for *a* × *CL* crosses, a drop of bacterial suspension on DSDM agar being simultaneously inoculated with *a* and *CL* amoebae. Small plasmodia which formed were separately subcultured to SDMS agar and allowed to grow into large vigorous plasmodia. These were then tested for their fusion behaviour against appropriate testers. No crossed plasmodia were detected. In a modified procedure, an excess of *a* amoebae was inoculated together with *CL* amoebae, and at daily intervals until plasmodia appeared, additional bacteria were added and the whole gently remixed with a wire loop. This procedure should have avoided local concentrations of mt_h amoebae but again no crossed plasmodia were found.

Observations of heterothallic crosses suggest that a period of growth may be necessary before the cells become competent to mate. Consequently spots of *a* and *CL* amoebae were placed at various distances apart (1–3 cm) on a lawn of bacteria. The growing areas touched after various periods of growth. Where the growing edges met before plasmodia had formed in the *CL* colony, plasmodia subsequently formed at the junction were separately subcultured and their fusion behaviour tested. No crossed plasmodia were found.

Crosses between amoebae of strains *a* and *CL* were ultimately achieved using a derivative line of *CL* amoebae showing delayed plasmodium formation (*CLd*). The *CLd* line was derived from *CL* by repeated subculture of amoebae in plasmodium-forming conditions. Approximately 10^5 *CL* amoebae were inoculated together with a drop of bacterial suspension onto DSDM agar. After 4–5 days when plasmodia had formed in the lawn of amoebae, the plate was flooded with 2 ml of water and residual amoebae gently resuspended. The amoebal suspension was spread, together with a bacterial suspension over the surface of several DSDM

plates (0.1 ml/plate) and the process repeated. After 12 such steps the residual amoebae were subcultured on LIA in order to obtain a large amoebal population. Cells of this population were then plated on 200 plates of DSDM agar with bacteria in order to score the plasmodia-forming capacity of plaques which they produced. After 7 days' incubation, approximately 10% of plaques showed well developed plasmodia. The majority of plaques showed delayed plasmodium formation, having only one or two small plasmodia at their centre. Amoebae from 50 of these plaques taken at random were subcultured and plasmodia obtained from them were found to spore readily. The amoebae from several of the 50 plaques were individually test-mated with *a* amoebae and were found to produce crossed plasmodia. One such clone of amoebae, designated *CLd* (*CL* delayed plasmodium formation) was used for subsequent crosses with heterothallic strains. This line, *CLd* produces plasmodia in clones of amoebae but does so only after a characteristic delay (7–10 days). *CLd* and *CL* plasmodia are indistinguishable in other characteristics. They both exhibit the same fusion behaviour, have the haploid nuclear DNA content (Cooke & Dee, 1974), sporulate readily and produce progeny amoebae which form plasmodia in plaques with 100% efficiency. No delay is observed in plasmodium formation by *CLd* progeny clones.

(iii) Construction of heterothallic strains isogenic with *CL*

The heterothallic mating type alleles mt_1 and mt_2 from the Wisconsin 1 isolate (Dee, 1973) were chosen for introduction into the *CL* (*CLd*) genetic background. This isolate was chosen primarily because like *Colonia* derivatives, it carries the plasmodial fusion alleles *fusA2* and *fusB1* and it therefore seems likely that the strains are not too distantly related. Secondly progeny of crosses between Wisconsin 1 strains and *Colonia* derivatives can be genetically analysed without complication as demonstrated by Wheals (1973).

The particular mt_1 and mt_2 Wisconsin 1 strains chosen for initial crosses to *CLd* were *a* ($mt_1 fusA1 fusB1$) and *LU1* ($mt_2 fusA1 fusB1$). The crosses were set up on DSDM agar and the crossed plasmodia (*a* × *CLd*)1 and (*LU1* × *CLd*)1 were identified on the basis of their fusion behaviour as described in (ii) above. Progeny clones were classified for mating type, the plasmodial fusion alleles and *sax*. The results are shown in Tables 1 and 2.

In the cross (*a* × *CLd*)1 the ratio $mt_1:mt_1$ showed significant deviation from 1:1 at the 1% level and the reason for this is not clear. However the allele ratio $sax^+:sax^-$ was 1:1 and the ratio of recombinants:parentals for *mt* and *sax* was also 1:1. This is conclusive evidence that the plasmodium (*a* × *CLd*)1 resulted from a cross between *a* and *CLd* amoebae and that all progeny analysed came from meiosis in diploid heterozygous nuclei.

The progeny clone *LU173* ($mt_1: fusA1: fusB1: sax^+$) was selected from the progeny of (*a* × *CLd*)1 for backcrossing to *CLd*. It was necessary to select an mt_1 clone which carried the *fusA1* allele in order that crossed plasmodia might be detected in this and future backcrosses to *CLd*. It is possible that, by selecting progeny clones having the mt_1 and *fusA1* alleles from the Wisconsin 1 parent,

whole linkage groups from that isolate may be maintained in the selected progeny. However, this was avoided for the *fusA1* linkage group by selecting a recombinant *fusA1 sax⁺* progeny clone. A similar procedure could not be adopted for *mt₁* as there are no known markers in the same linkage group.

A crossed plasmodium (*LU173* × *CLd*)₄, obtained from the cross *LU173* × *CLd* was spored and a *mt₁fusA1fusB1* clone again selected for backcrossing to *CLd*. Three successive backcrossing steps were carried out (Table 3) and a *mt₁fusA1fusB1 sax⁺* progeny clone, LU648, was selected from the 5th generation. This strain,

Table 1. *Analysis of progeny of the plasmodium (a × CLd)1*

		<i>fusA1</i>	<i>fusA2</i>	Total			
* <i>mt₁</i>	<i>sax⁺</i>	5	24	29	}	54 <i>mt₁</i>	}
	<i>sax⁻</i>	—	—	25			
<i>mt_h</i>	<i>sax⁺</i>	3	37	40	}	86 <i>mt_h</i>	}
	<i>sax⁻</i>	—	—	46			
				140			71 <i>sax⁻</i>

* For scoring *sax* and *fus* plasmodia were derived from *mt₁* progeny amoebae by crossing with amoebae of the genotype: *mt₂; fusA1; fusB1; sax⁻*.

Recombination frequency between *fusA* and *sax* = $\frac{8}{69} \times 100 = 11.6\%$.

Table 2. *Analysis of progeny of the plasmodium (LU1 × CLd)*

		<i>fusA1</i>	<i>fusA2</i>	Total
* <i>mt₂</i>	<i>sax⁺</i>	1	30	31
	<i>sax⁻</i>	—	—	34
<i>mt_h</i>	<i>sax⁺</i>	—	—	} 47
	<i>sax⁻</i>	—	—	

* For scoring *sax* and *fus* plasmodia were derived from *mt₂* progeny amoebae by crossing with amoebae of the genotype: *mt₁; fusA1; fusB1; sax⁻*.

which could be theoretically attributed with 97% *CL* genetic background, was selected for use as a *mt₁* strain 'isogenic' with *CL* (*mt₁; CL*).

In order to produce a *mt₂; CL* strain, the recombinant progeny clone *LU418mt₂fusA1fusB1* from the cross (*LU1* × *CLd*)₁ was selected for backcrossing to *CLd*. However, all attempts to isolate crossed plasmodia from *LU418* × *CLd* failed. Of 114 plasmodia tested, all fused with tester plasmodia of fusion group V indicating that they had formed directly from *CLd* amoebae. Wheals (1970) also reported difficulty in crossing *mt₂* with *Colonia* amoebae and previously Dee (1966) had noted strain-dependent low crossing frequencies where strains were closely related, for example, parent and progeny. An alternative method of producing an *mt₂* strain isogenic with *CL* was therefore adopted. Amoebae of the strain *LU418*, which could theoretically be attributed with approximately 50% *CL* genetic background,

were crossed with *LU523* amoebae; *mt*₁ (75% *CL*) (Table 4). An *mt*₂ progeny clone *LU586* was selected and crossed with the next available *mt*₁*CL* strain, *LU574mt*₁ (87% *CL*) (Table 4). After 3 further intercrosses an *mt*₂ clone, theoretically attributable with 94% *CL* background, was isolated. This strain *LU688* (*mt*₂*fusA1 fusB1 sax*⁺) was selected for use as an *mt*₂*CL* strain.

Table 3. Construction of *mt*₁; *CL* strain *LU648*

Cross	Selected crossed plasmodium	Number of progeny clones analysed*	Selected progeny clone†	Theoretical proportion of background attributable to <i>CL</i> genes (%)
<i>a</i> × <i>CLd</i>	(<i>a</i> × <i>CLd</i>)1	140	<i>LU173</i>	50
<i>LU173</i> × <i>CLd</i>	(<i>LU173</i> × <i>CLd</i>)4	25	<i>LU523</i>	75
<i>LU523</i> × <i>CLd</i>	(<i>LU523</i> × <i>CLd</i>)15	10	<i>LU574</i>	87
<i>LU574</i> × <i>CLd</i>	(<i>LU574</i> × <i>CLd</i>)5	30	<i>LU624</i>	93.5
<i>LU624</i> × <i>CLd</i>	(<i>LU624</i> × <i>CLd</i>)10	30	<i>LU648</i>	97

* All progeny clones were classified for *mt*; *fus*; *sax* and results agreed with expectations.

† Genotype of selected progeny clones: *mt*₁; *fusA1*; *fusB1*; *sax*⁺.

Table 4. Construction of *mt*₂; *CL* strain *LU688*

Cross	Selected crossed plasmodium	Number of progeny clones analysed	Selected progeny clone*	Theoretical proportion of background attributable to <i>CL</i> genes (%)
<i>LU1</i> × <i>CLd</i>	(<i>LU1</i> × <i>CLd</i>)1	112	<i>LU418</i>	50
<i>LU418</i> × <i>CLd</i>	None	—	—	—
<i>LU523</i> † × <i>LU418</i>	<i>LU523</i> × <i>LU418</i>	10	<i>LU586</i>	62.5
<i>LU574</i> † × <i>LU586</i>	<i>LU574</i> × <i>LU586</i>	10	<i>LU599</i>	75
<i>LU624</i> † × <i>LU599</i>	<i>LU624</i> × <i>LU599</i>	10	<i>LU635</i>	84
<i>LU648</i> † × <i>LU635</i>	<i>LU648</i> × <i>LU635</i>	10	<i>LU677</i>	90.5
<i>LU648</i> † × <i>LU677</i>	<i>LU648</i> × <i>LU677</i>	10	<i>LU688</i>	94

* Genotype of selected progeny clones: *mt*₂; *fusA1*; *fusB1*; *sax*⁺.

† *mt*₁ amoebal clones derived from the backcross series shown in Table 3.

(iv) Isolation of plasmodial mutants in strain *CL*

Exponentially growing cultures of *CL* amoebae were mutagenized, reincubated on growth medium to allow fixation and expression of mutations, and then plated at various dilutions on DSDM agar. Separate plaques which developed gave rise to plasmodia after a few days. Plasmodia derived from individual plaques were separately subcultured to complete medium (SDMS agar) and allowed to form large vigorous plasmodia. The latter were then tested for growth on minimal medium (DM-1), growth at 31 °C on complete medium and utilization of maltose as an alternative carbon source. Plasmodia which failed to grow on DM-1 were tested for growth on DM-1 supplemented with 13 amino acids (DM-3) or with a

combination of amino acids, bases and vitamins. Further tests to identify specific requirements were made.

A total of 5000 plasmodia obtained after UV or NMG mutagenesis of *CL* amoebae have been screened in this way. One lysine requiring auxotroph, *CL348* (*lys*⁻) and one temperature-sensitive mutant, *CL2039* were identified (Table 5). In both cases ten progeny lines of the mutant plasmodia were isolated and found to express the same mutant characteristics as the parent. Both progeny amoebae and plasmodia of *CL2039* were found to be temperature sensitive.

In order to cross mutants isolated in strain *CL* with isogenic *mt*₁ or *mt*₂ strains it was necessary to derive variants of the mutant strains showing delayed plasmodium formation. A strain *CL348d* was derived from *CL348* by the method described in (ii) above.

Table 5. Isolation of mutants of strain *CL/CLd*

Amoebae used for mutagenesis	Mutagen	Number of plasmodia screened	Mutant strains isolated	Phenotype
<i>CL</i>	UV (+ caffeine)	2.5 × 10 ³	<i>CL348</i>	Lysine requiring (<i>lys</i> ⁻)
<i>CL</i>	NMG	2.5 × 10 ³	<i>CL2039</i>	No growth at 29 °C (<i>ts</i>)
<i>CLd</i>	NMG	7.5 × 10 ³	<i>CL5001</i>	Leucine requiring (<i>leu</i> ⁻)

(v) Isolation of plasmodial mutants in strain *CLd*

CLd amoebae were mutagenized as described in (iv) but were plated dilutely on LIA after post-mutagenesis incubation. Amoebae from single plaques were transferred with a wire loop to a 'puddle' of thick bacterial suspension on dilute DM-1 plates (DDM-1).

After approximately 2 weeks' growth in these conditions *CLd* amoebae formed plasmodia which migrated from the inoculation spot to the peripheral regions of the plate. Plasmodia which migrated but failed to grow under these conditions were 'rescued' by subculturing to SDM and tested for nutritional requirements. At the same time any remaining amoebae from the 'puddle site' were rescued and inoculated onto fresh LIA plates.

Using this method a leucine requiring mutant strain *CL5001d* (*leu*⁻) has been obtained (Table 5).

(vi) Plasmodial mutagenesis

We have recently attempted to obtain mutants by exposing *CL* plasmodia to NMG during the S period, allowing them to spore after further growth and subsequently screening plasmodia obtained from progeny amoebal clones for nutritional mutants. A number of plasmodia unable to grow on DM-1 have been isolated but so far it has not been possible to identify specific requirements.

(vii) *Mutant analysis*

Amoebae of the mutant strains *CL348d* and *CL5001d* were mated with the heterothallic *mt*₁*CL* strain *LU648* and crossed plasmodia selected as previously described. Both *lys*⁻ and *leu*⁻ were found to be recessive. Progeny clones were classified for mating type and plasmodia derived from the *mt*_h progeny clones were tested for fusion type and nutritional requirement. Tables 6 and 7 show the analysis of progeny from the crossed plasmodia (*LU648* × *CL348d*)106 and (*LU648* × *CL5001d*)2 respectively.

Table 6. *Analysis of progeny of the plasmodium (LU648 × CL348d)106*

		<i>lys</i> ⁺	<i>lys</i> ⁻	Total		
<i>mt</i> ₁	{	—	—		} 49 <i>mt</i> ₁	
<i>mt</i> _h		<i>fusA1</i>	10	17		27
		<i>fusA2</i>	10	11		21
		20	28			

Plasmodia were tested for nutritional requirement on DM-1 and DM-1 + lysine.

Table 7. *Analysis of progeny of the plasmodium (LU648 × CL5001d)2*

		<i>leu</i> ⁺	<i>leu</i> ⁻	Total		
<i>mt</i> ₁	{	—	—		} 92 <i>mt</i> ₁	
<i>mt</i> _h		<i>fusA1</i>	20	20		40
		<i>fusA2</i>	16	28		44
		36	48			

Plasmodia were tested for nutritional requirement on DM-1 and DM-1 + leucine.

4. DISCUSSION

The derivation of strains *CL* and *CLd* and the construction of isogenic heterothallic strains described in this paper have provided a relatively simple system for the isolation and genetic analysis of plasmodially expressed mutants of *P. polycephalum* within a uniform genetic background. To test this system, two auxotrophic mutants were isolated in strain *CL* (*CLd*) and were shown to be due to single gene mutations. In the present work only the *mt*_h progeny of the crosses were analysed as this provides a rapid means of determining that a mutant is due to a single gene defect; unless that mutation is linked to *mt*, in which case deviation from a 1:1 allele ratio would be found amongst the *mt*_h progeny.

The isolation of further plasmodial mutants would be greatly facilitated by enrichment and other selective techniques and work is at present in progress in this and other laboratories to develop such methods.

It is envisaged that *CL* and *CLd* will both be of value for future mutant isolation work. In enrichment techniques which require the production of very large

numbers of clonally formed plasmodia, the use of a strain such as *CL* which produces plasmodia in plaques is essential. The isolation of mutants blocked in the amoebal-plasmodial-transition (*apt* mutants, Wheals, 1973) also requires the use of such a strain. However, *CLd* has advantages for the isolation of mutants expressed in amoebae. Plasmodia carrying such mutations can easily be produced from the amoebal clones. *CLd* also has the advantage that mutants isolated in this strain can be crossed directly with *mt*₁*CL*.

As reported in the results, plasmodia which arise from *CLd* amoebae give rise to progeny amoebae which no longer exhibit the delay in plasmodia formation shown by *CLd*; all progeny produce plasmodia in 100 % of plaques without delay. However when *CLd* amoebae are crossed with heterothallic strains all the *mt*_n progeny amoebae show a delay in plasmodium formation similar to that of *CLd*. One hypothesis is that the delay is due to a cytoplasmic factor and that this is lost from a small proportion of *CLd* amoebae during prolonged growth. These cells then undergo plasmodium formation in a similar manner to *CL* amoebae and give normal *mt*_n progeny. Where *CLd* is used in crosses with heterothallic strains then the hypothesis would suggest that mating occurs between the two strains as in heterothallic crosses without the loss of the cytoplasmic factor. Thus the factor would be inherited by progeny amoebae and would cause a delay in the formation of plasmodia within *mt*_n clones. The results are however, also compatible with delayed plasmodium formation being due to a nuclear gene mutation at or closely linked to the *mt* locus. Clonal plasmodium formation would in this case be due to reversion, though this would seem to require a mutation rate higher than is normally observed at the *mt* locus.

At present the events leading to clonal plasmodium formation by *mt*_n amoebae are not understood although it is known that there is no nuclear fusion (Cooke & Dee, 1974). Work is at present in progress in this laboratory to determine whether fusion of amoebae is involved.

In addition to the *mt*₁*CL* and *mt*₂*CL* strains derived in the present work, *mt*₃ and *mt*₄ strains partially isogenic with *CL* have been constructed by Adler and Holt (submitted for publication). The *mt*₃ and *mt*₄ alleles are derived from the Indiana isolate of *P. polycephalum* and the strains are known to differ from *CL* at a new locus controlling plasmodial fusion (*fusC*). Adler and Holt reported that it is possible to mate *mt*₃ and *mt*₄ amoebae directly with *CL* amoebae and to obtain crossed plasmodia as judged by their failure to fuse with *CL* plasmodia. Though this direct mating facility decreased during their series of backcrosses of *mt*₃ to *CL*, they state, however, that crossed plasmodia may still be obtained from such matings. Undoubtedly all four heterothallic *mt* strains partially isogenic with *CL* which have been constructed will be of value for the future analysis of mutants isolated in the *CL* strain of *P. polycephalum*.

We wish to thank Mrs Jennifer Foxon and Miss Eileen Pott for their invaluable help in the isolation of mutants and some of the stages of strain construction. We are especially indebted to Ned Holt and Paul Adler for keeping us informed of their results and also to them and several other colleagues for helpful criticism. D.J.C. was supported during the course of this work by an S.R.C. Studentship which we acknowledge with thanks.

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