Genet. Res., Camb. (1974), **23**, *pp.* 307–317
With 3 *text-figures*
Printed in Great Britain

Plasmodium formation without change in nuclear DNA content in *Physarum polycephalum*

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(*Received* 21 *January* 1974)

SUMMARY

The Colonia isolate of *Physarum polycephalum* produces plasmodia within amoebal clones. Wheals demonstrated genetically that amoebae of the *C*50 strain of this isolate, when crossed with heterothallic amoebae, yielded recombinant progeny. He concluded that nuclear fusion and meiosis occurred in these crosses and suggested that nuclear fusion was also involved in plasmodia formation in clones. He thus designated the strain 'homothallic'.

In the present work genetic evidence is presented which indicates that the Colonia strain *CL*, when crossed with heterothallic strains, also yields recombinant progeny and thus undergoes nuclear fusion and meiosis. Microdensitometric measurements of nuclear DNA content are reported which indicate that *CL* amoebae are haploid like heterothallic amoebae, and crossed plasmodia are diploid. However, clonally formed *CL* plasmodia were found to have the same G_2 nuclear DNA content as *CL* amoebae. This observation excludes the possibility of nuclear fusion when plasmodia form within clones of *CL* amoebae and therefore the strain cannot be homothallic. Two alternatives, apogamy and coalescence, are proposed as the most likely mechanisms for clonal plasmodium formation in strain *CL*.

1. INTRODUCTION

The Colonia isolate of *Physarum polycephalum* differs from heterothallic isolates in being able to complete the life-cycle within single clones. Uninucleate amoebae give rise to a macroscopic multinucleate plasmodium which produces spores under appropriate conditions. The spores hatch to release uninucleate amoebae capable of repeating the life-cycle.

The Colonia isolate was originally described by Von Stosch, Van Zul-Pischinger & Dersch (1964), who suggested that the strain was 'homothallic' but did not give detailed evidence. Wheals (1970) genetically analysed the progeny of crosses between a Colonia derivative strain (*C*50) and heterothallic strains and showed that the ability of *C*50 amoebae to form plasmodia within clones was determined by a single allele at the mating-type locus (*mt*) which he designated mt_h. His genetic results indicated that both nuclear fusion and meiosis occurred in such crosses. Wheals therefore suggested that within clones of Colonia amoebae, plasmodial development also involved nuclear fusion, i.e. that the strain was homothallic. If this is so, nuclei of Colonia plasmodia would be expected to have

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twice the ploidy of the amoebae. The aim of the present work was to test this prediction by comparative measurements of nuclear DNA content of amoebae and plasmodia derived from the Colonia strain *CL* and representative heterothallic strains.

The average nuclear DNA content at different stages in the life-cycle of heterothallic strains of *P. polycephalum* has been measured by Mohberg & Rusch (1971), who concluded that in these strains, amoebae were haploid and plasmodia diploid. Therrien (1966), on the basis of microdensitometric measurements of nuclear DNA content in *Didymium nigripes*, concluded that his strain was homothallic, haploid amoebae fusing to produce diploid zygotes and plasmodia. Kerr (1968) discussed Therrien's results and questioned his conclusions. She reported chromosome counts on strains of *D. nigripes* and discussed time-lapse cinematographic studies by N. Kerr (1967). S. Kerr (1968) concluded that there was no ploidy level difference between amoebae and plasmodia and that nuclear and cytoplasmic fusion were not necessary for plasmodia formation.

2. MATERIALS AND METHODS

(i) *Strains.* The heterothallic amoebal strains *a* and *i* have been previously described (Dee, 1966). Strains *CL* (Colonia Leicester) and *CL*d (*CL* delayed plasmodia formation) were derived by successive cloning from *C*50 (Wheals, 1970) and their isolation will be described elsewhere (Cooke, manuscript in preparation). Under appropriate conditions *CL* amoebae form plasmodia in 100% of amoebal plaques and when suitably subcultured such plasmodia are able to complete the life-cycle. *CL*d amoebae also produce plasmodia within individual clones but do so only after a characteristic delay (7–10 days). *LU*523 is a heterothallic (mt_1) strain derived by backcrossing (to *CL*d) a mt_1 progeny clone from the cross *a* × *CL*d.

(ii) *Loci. mt*: mating type. Alleles mt_1 mt_2: heterothallism (Dee, 1966); mt_h: plasmodia formation within amoebal clones ('homothallism'; Wheals, 1970).

f and *n*: plasmodial fusion type (Poulter & Dee, 1968; Poulter, 1969). Identity at both *f* and *n* loci is a prerequisite for plasmodial fusion.

sax: sensitivity to axenic medium (Poulter, 1969; Wheals, 1973). Plasmodia homozygous for sax^- fail to grow when subcultured to axenic medium.

(iii) *Genotypes of amoebal strains. a*: mt_1; f_1; n_1; sax^-. *i*: mt_2; f_2; n_2; sax^+. *CL* (and *CL*d): mt_h; f_2; n_1; sax^+. *LU*523: mt_1; f_1; n_1; $sax+$.

(iv) *Cultural conditions.* Amoebae were maintained in two-membered culture with *Escherichia coli* at 26 °C on liver infusion agar (LIA) containing 1 g Oxoid liver infusion agar powder per litre of 2% agar. *CL* amoebae may be maintained on LIA plates without plasmodia formation in plaques by (*a*) regular subculturing every 3–4 days or (*b*) transferring to 4 °C after 4 days incubation. All plasmodia were routinely cultured at 26 °C on a semidefined agar medium (SDM) (Dee & Poulter, 1970). Production of spores, spore plating and isolation of progeny clones were carried out by methods previously described (Wheals, 1970).

(v) *Plasmodium formation.* Plasmodium formation by amoebae derived from a

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single clone was achieved by inoculating a dilute (6 %) SDM agar plate with approximately 10^3 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) *Nuclear isolation*. A modified version of methods previously described by Mohberg & Rusch (1971) was used for isolation of plasmodial nuclei. Plasmodia were harvested in G_2 phase, the time in the mitotic cycle being determined by phase-contrast microscopic observation of glycerol/ethanol fixed smears (Mittermayer, Braun & Rusch, 1965). Five plasmodia 6–7 cm in diameter were carefully scraped from SDM agar into 200 ml of ice-cold medium A: 250 mM sucrose, 20 mM Tris (pH 7·2 with HCl), 20 mM-$MgCl_2$, 10 mM Mercaptoethanol, 0·1 % (w/v) Triton X-100. The suspension was homogenized in an MSE Ato-Mix blender coupled to a Berco variable resistance transformer. Crossed plasmodia were homogenized at a nominal 50 % of mains voltage (240 V) half speed for 15 sec and set at full speed for 30 sec. To obtain clean preparations from clonally formed plasmodia however, it was found necessary to homogenize at full speed for 1 min. After standing in ice for 5 min to allow froth to settle the homogenate was filtered through a cotton-wool milk filter pad sandwiched between two 7·5 in. fabric milk filters (Grant & Poulter, 1973). The filtered homogenate was decanted into four 50 ml conical glass tubes and centrifuged for 10 min (0 °C) at 2000 rev/min in a Sorval HS-4 rotor. The pellet was resuspended in 100 ml of medium B (as medium A but omitting Triton X-100), and centrifuged as described above. The final pellet was resuspended on 0·3 ml medium B, frozen in liquid N_2 and stored at -20 °C.

To ensure that the amoebae used for nuclear isolation were in logarithmic phase (therefore mostly in G_2) and not encysted (G_1) (Mohberg & Rusch, 1971), LIA plates were inoculated with approximately 50 amoebae per plate and 0·1 ml bacterial suspension. When large plaques had developed (after 4–5 days incubation) a further 0·2 ml of bacterial suspension was added, the plates respread and incubated for 2 days. Harvesting these plates by flooding with 5 ml ice-cold water and gentle scraping with a glass spreader yielded approximately 5×10^6 amoebae/plate with no visible cysts.

Amoebal suspensions, essentially freed of bacteria by repeated washing in ice-cold water (Mohberg & Rusch, 1971), were then subjected to the nuclear isolation procedure outlined above. Homogenization was at full speed for 1 min and centrifugation at 2200 rev/min. Phase-contrast microscopic observation of the final suspension showed a preparation which was not distinguishable from isolated plasodial nuclei. However, after Feulgen staining (see below) it became apparent that the amoebal 'nuclear' preparations consisted of whole amoebae with only the nucleus being stained. Stain intensity in the cytoplasm was determined and found to be negligible. The cause of the apparently identical appearance of whole

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amoebae and isolated plasmodial nuclei was found to be the presence of Triton in medium A. Amoebal cells simply suspended in medium A (or 0·1% Triton alone) were found to round off immediately and assumed the characteristic appearance of isolated plasmodial nuclei and this was not reversed when the cells were transferred to medium B.

(viii) *Estimation of nuclear DNA content.* Preparations were rapidly thawed and a drop of each air-dried on a slide before being fixed in acetic alcohol for 1 h. Specimens were stained by the Feulgen method (Darlington & La Cour, 1962) with the modification that hydrolysis was by 5 N-HCl at room temperature for 45 min (Itakawa & Ogura, 1954). Stain intensity was measured with a Vickers M 85 Scanning Microdensitometer (Vickers Instruments Ltd). Fifty nuclei of each preparation were each scanned once using the following settings: slit width 20, wavelength 55 and spot size 2.

3. RESULTS

(i) *Identification of crossed plasmodia from heterothallic* × mt_h *matings*

In a cross involving heterothallic and mt_h amoebae two types of plasmodia are possible, namely those arising directly from mt_h amoebae and those resulting from crossing between the heterothallic and mt_h amoebae. To identify the two classes of plasmodia formed in this type of cross the plasmodial fusion system was used (Poulter & Dee, 1968). Thus a plasmodium which resulted from a cross between amoebae of the genotypes $mt_1f_1n_1$ (*a*) and $mt_hf_2n_1$ (*CL*d) would have the genotype $f_1f_2n_1n_1$ (fusion group III) whereas plasmodia formed in clones of *CL*d (or *CL*) amoebae fuse with tester plasmodia of fusion group V ($f_2f_2n_1n_1$). 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.

To set up a cross such as *a* × *CL*d, a drop of bacterial suspension on DSDM agar was simultaneously inoculated with *a* and *CL*d amoebae. As soon as individual plasmodia became visible (3–5 days) several were separately subcultured to SDMS agar and allowed to grow into large vigorous plasmodia which were then tested for their fusion behaviour against appropriate testers. It was found that each plasmodium could be unambiguously assigned to either fusion group V or fusion group III. The plasmodia could therefore be classified as having arisen either directly from *CL*d amoeabe or by mating between *a* and *CL*d amoebae (i.e. crossed).

(ii) *Comparison of nuclear DNA content of amoebal strains* a, CL, CL*d and plasmodia* a × i, CL, CL*d, and* a × CL*d*

One crossed plasmodium (*a* × *CL*d)1 identified as described above was selected for estimation of nuclear DNA content. Genetic analysis of the progeny of (*a* × *CL*d)1 (see below) confirmed that this plasmocium had resulted from the sexual fusion of *a* and *CL*d amoebae.

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Fig. 1 shows measurements of nuclear DNA content of Feulgen-stained log. phase amoebae of strains *a*, *CL*, *CL*d (see Materials and Methods) and isolated nuclei of plasmodia *CL*, *CL*d, *a* × *i* and (*a* × *CL*d)1. The *CL* and *CL*d plasmodia used were produced in single clones.

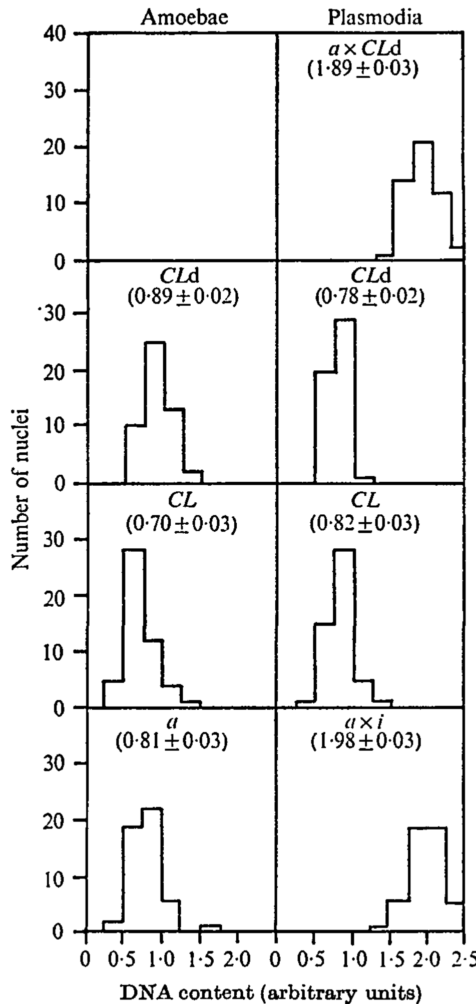

Fig. 1. Microdensitometric estimation of amoebal and plasmodial nuclear DNA content. All samples were Feulgen stained as a single batch. Mean values and standard errors are shown in parentheses.

It can be seen that, as expected, *a* × *i* plasmodial nuclei have twice the DNA content of *a* amoebal nuclei, agreeing with the conclusion of Mohberg & Rusch (1971) that heterothallic amoebae are haploid and plasmodia diploid.

The crossed plasmodium (*a* × *CL*d)1 also has a nuclear DNA content twice that of *a* (and *CL*d) amoebae. However, the *CL* and *CL*d plasmodia have a nuclear DNA content approximately the same as that of *CL*, *CL*d and *a* amoebae. These data strongly suggest that:

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(*a*) *CL*d amoebae can undergo cell and nuclear fusion with heterothallic amoebae to produce diploid plasmodia.

(*b*) *CL*d (and *CL*) plasmodia are haploid and plasmodia formation within clones of this strain of *P. polycephalum* is accomplished without nuclear fusion.

(iii) ($a \times$ CL*d*)1 *progeny analysis*

The plasmodium (a × *CL*d)1 was allowed to spore and 140 progeny amoebal clones isolated. Analysis of these progeny clones yielded the results shown in Table 1. The ratio $mt_h:mt_1$ 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* × *CL*d)1 resulted from a cross between *a* and *CL*d amoebae and that all progeny analysed arose from meiosis in diploid heterozygous nuclei.

Table 1. *Progeny analysis of the plasmodium* (a × CL*d*)1

	sax^+	sax^-	Total
mt_1	29	25	54
mt_h	40	46	86
Total	69	71	140

Parental classes		Recombinant classes	
$mt_1\ sax^-$	25	$mt_1\ sax^+$	29
$mt_h\ sax^+$	40	$mt_h\ sax^-$	46
Total	65		75

mt_1 amoebae: failed to form plasmodia within clones. Plasmodia produced when crossed to mt_2. sax^- tester amoebae.

mt_h amoebae: produced plasmodia within clones. sax^- plasmodia: plasmodia (produced in tests of mating type) which failed to grow when subcultured to axenic medium (SDMS) were classified as sax^-.

(iv) *Comparison of nuclear DNA content in successive generations of* mt_h *and* mt_1 *amoebae and plasmodia*

To test the conclusions given in (ii) above, measurements of nuclear DNA content were made on a further eight strains of amoebae and plasmodia which were derived as shown in Table 2. Amoebae of strain *CL*d were mixed with amoebae of the heterothallic mt_1 strain *LU*523. Plasmodia isolated from the cross plate were found to be of two fusion types – those which fused with a tester plasmodium of fusion group V, as expected if they were derived directly from *CL*d amoebae, and those which fused with a tester plasmodium of group III, as expected if they were formed by mating between *CL*d and *LU*523 amoebae. One plasmodium of each of these types was chosen as a representative plasmodium from which progeny amoebae and plasmodia were isolated.

The nuclear DNA content of all the representative strains named in Table 2 was estimated and the results are shown in Figs. 2 and 3. Again all material was

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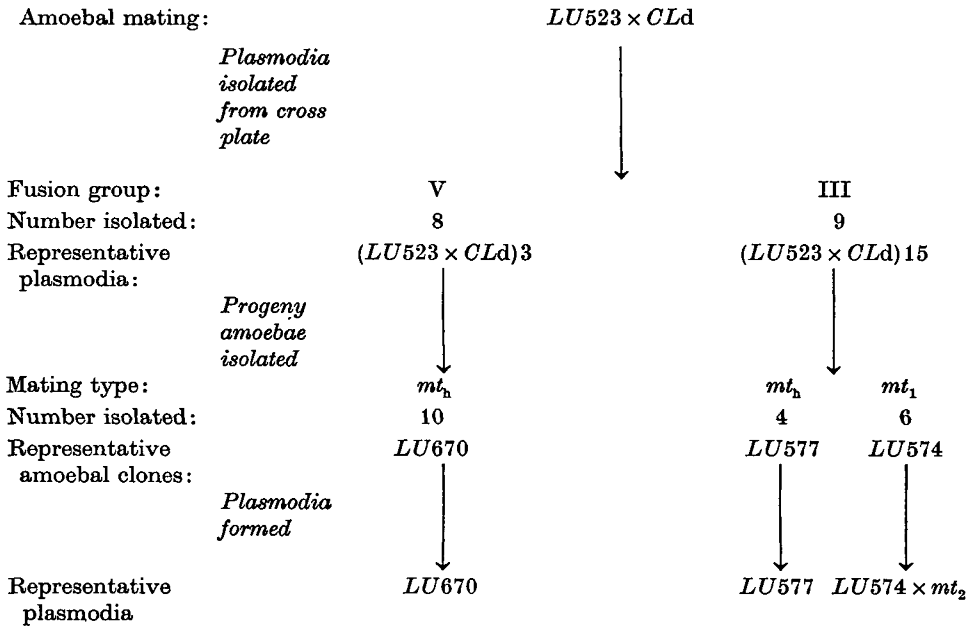
Feulgen stained as a single batch to enable valid comparisons to be made. Also included in the staining series as control samples were nuclei of the *CL* plasmodium and the $a \times i$ plasmodium from (ii) above.

Table 2. *Derivation of successive generations of amoebae and plasmodia for estimation of nuclear DNA content*



Measurements of nuclear DNA content were made on the named representatives of each class (see Figs. 2 and 3). As indicated, these representatives of each generation were also used to produce the next generation. Amoebae which gave rise to plasmodia within clones were classified as mt_h. Amoebae which only gave plasmodia when test mated with mt_2 amoebae were classified as mt_1.

The histograms Fig. 2 (*a*)–(*c*), show the measurements for the representative group V plasmodium, a progeny amoebal clone and the plasmodium which this clone produced. Both plasmodia have nuclear DNA contents close to that of the amoebal strain and similar to that of the control *CL* plasmodium (Fig. 2*d*), supporting the previous conclusion that plasmodia formed in clones of mt_h amoebae are haploid.

Fig. 3(*a*) shows the nuclear DNA content of the plasmodium (*LU*523 × *CL*d)15 which was classified as crossed on the basis of its fusion behaviour. As expected the plasmodium appears to be diploid, its DNA content being similar to that of the control ($a \times i$) (Fig. 2*e*).

Both the mt_h (*LU*577) and mt_1 (*LU*574) progeny amoebae from this crossed plasmodium have the haploid DNA content (Fig. 3*b*, *d*). In agreement with the other results, the plasmodium formed in clone *LU*577 has a haploid DNA content (Fig. 3*c*) while the plasmodium *LU*574 × mt_2 (Fig. 3*e*) has a diploid value. All the results are therefore consistent with the conclusions summarized in (ii).

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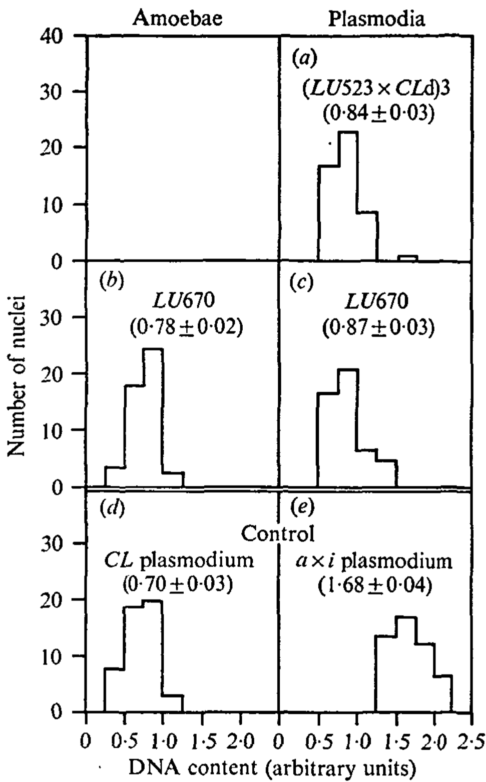

Fig. 2

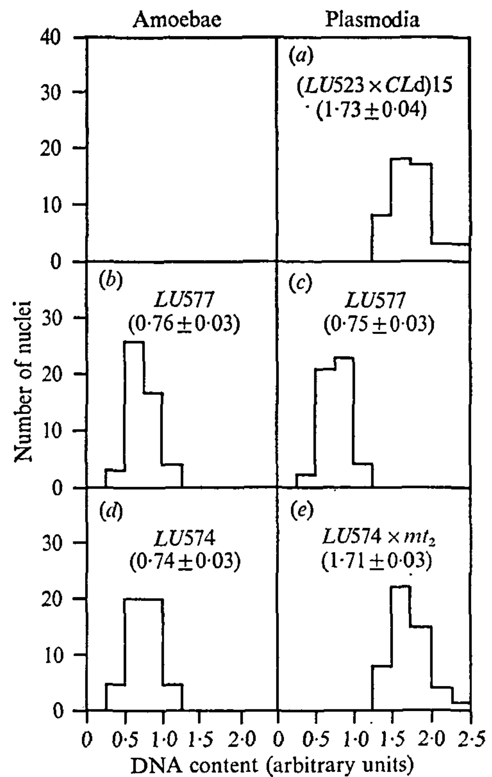

Fig. 3

Fig. 2. Microdensitometric estimation of amoebal and plasmodial nuclear DNA content. See Table 2 for derivation of the *LU* strains. All samples were stained together with those shown in Fig. 3 as a single batch. Mean values and standard errors are shown in parentheses.

Fig. 3. Microdensitometric estimation of amoebal and plasmodial nuclear DNA content. See Table 2 for derivation of the *LU* strains. All samples were stained together with those shown in Fig. 2 as a single batch. Mean values and standard errors are shown in parentheses.

4. DISCUSSION

The data presented show that amoebae and plasmodia of the *CL* strain of *P. polycephalum* have the same nuclear DNA content and this content is half that of crossed plasmodia. Yemma & Therrien (1972) observed in 'selfing clones' of the usually heterothallic myxomycete *Didymium iridis* that amoebae and plasmodia had the same nuclear DNA content (2C), a value which was half that of crossed plasmodia (4C). They also observed that in all cases sporangial nuclei contained the 4C amount. They proposed that amoebae were haploid and in G_2 phase, that crossed plasmodia were diploid and in G_2 phase and that selfed plasmodia were also diploid but were in an extended G_1 phase. This does not seem to be the case for *CL* plasmodia because Sudbery (personal communication) has measured [^{3}H]-thymidine incorporation in *CL* plasmodia and has found that DNA synthesis

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commences immediately after mitosis in this strain and is complete within 3 h. These results indicate that as in heterothallic strains of *P. polycephalum* there is no G_1 phase in *CL* plasmodia. Plasmodia in the present work were not used for nuclear isolation until at least 3 h after mitosis had been completed and were therefore in G_2 phase. The present results are thus interpreted as indicating that there is no change in ploidy during plasmodium formation within clones of strain *CL*.

Mohberg *et al.* (1973) showed that plasmodia and spores of the Colonia strain *C*50 (see Materials and Methods) had the same average nuclear DNA content. In this and a previous study (Mohberg & Rusch, 1971) on *P. polycephalum*, heterothallic plasmodia in G_2 had twice the DNA content of G_2 amoebae and mature spores. Assuming that heterothallic amoebae and plasmodia have haploid and diploid nuclear DNA contents respectively, the present data strongly suggest that *CL* amoebae and plasmodia are both haploid. The average nuclear DNA contents estimated for *C*50 plasmodia and spores by Mohberg *et al.* (1973) were intermediate between those of heterothallic amoebae and plasmodia. The reason for this difference is not understood but is at present the subject of a joint investigation with Dr Mohberg in this laboratory. Chromosome counts (Mohberg *et al.* 1973) showed that plasmodia of *CL* (and other Colonia isolates) have 35–40 chromosomes whereas $a \times i$ plasmodia have predominantly nuclei with 45–50 or 75–80 chromosomes. Bradbury *et al.* (1973), however, reported a chromosome number of 22 for $a \times i$ plasmodia.

The fact that G_2 nuclei from amoebae and plasmodia of strain *CL* have the same DNA content suggests that plasmodia formation in this strain occurs without nuclear fusion. This is in contrast with plasmodia formation in heterothallic strains and in crosses between mt_h and heterothallic amoebae where nuclear fusion is indicated both by DNA measurements and by genetical results reported here and by Wheals (1970). Two alternative mechanisms for plasmodia formation within clones of *CL* amoebae seem possible:

(*a*) apogamy: a single amoebal cell developing into a plasmodium by repeated nuclear division without cell or nuclear fusion and without change in ploidy;

(*b*) coalescence: fusion of two or more genetically identical amoebae, without nuclear fusion, producing a dikaryotic cell which by repeated nuclear division gives rise to a plasmodium.

The present data do not allow one to distinguish between these alternatives although they do exclude the possibility in our strains of homothallic development since this requires nuclear fusion and consequent change in ploidy. Wheals (1970) found that a cross $mt_h f_2 \times mt_h f_4$ gave rise to hybrid plasmodia ($f_2 f_4$ fusion behaviour) and regarded this as additional evidence of nuclear fusion among mt_h amoebae. This could also be explained by coalescence of amoebae giving rise to heterokaryotic plasmodia. In attempts to repeat these observations we have tested 50 plasmodia obtained after mixing $mt_h f_1$ and $mt_h f_2$ amoebae for their fusion phenotype. Of these only one exhibited hybrid fusion behaviour, the remainder being clearly either f_1 or f_2 in fusion behaviour. These results alone do not give conclusive evidence of

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cell or nuclear fusion, but attempts to repeat and extend this preliminary investigation are in progress.

It is of great interest to understand the underlying processes involved in plasmodium formation since changes in morphology, nutrition and mode of nuclear division occur and it seems probable that differential gene activity is involved. It will also be of interest to understand the nuclear events associated with sporulation in Colonia isolates since meiosis occurs at this time in *P. polycephalum*. (Aldrich, 1967).

Previous work on the isolation and genetic analysis of mutants in the Colonia strain of *P. polycephalum* (Dee, 1973; Dee, Wheals & Holt, 1973; Wheals, 1973) was based on the assumption that clones of mt_h amoebae formed diploid homozygous plasmodia. However, the results of these studies agree equally well with the assumption that the plasmodia are haploid. The strains *CL* and *CL*d are now being used in several laboratories to isolate and analyse nutritional and temperature sensitive plasmodial mutants after mutagenesis of amoebae. The present results on the nuclear DNA content of *CL* (*CL*d) do not throw doubt on the value of these strains for the genetic analysis of *P. polycephalum* as discussed in detail by Dee (1973).

We are grateful to Mrs Jennifer Foxon for excellent technical assistance and to Dr A. E. Wheals and our colleagues in Leicester for helpful discussions during the preparation of this manuscript. One of us (D. J. Cooke) was in receipt of a Science Research Council Studentship and this is acknowledged with thanks.

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