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Viability of *Physarum polycephalum* Spores and Ploidy of Plasmodial Nuclei

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Amoebae of *Physarum polycephalum* carrying the *mt*h mating-type allele may differentiate into plasmodia in the absence of mating. Such plasmodia are haploid and, upon sporulation, produce mainly inviable spores. We have asked whether the viable spores arise from meiotic or mitotic divisions. Using a microfluorometric measurement of the deoxyribonucleic acid content of individual nuclei, we found the fraction of viable spores to be correlated with the proportion of rare, diploid nuclei contained in the generally haploid plasmodium. When homozygous diploid plasmodia were created by heat shocking, spore viability increased dramatically. We suggest that viable spores are produced via meiosis in *mt*h plasmodia, that the *mt*h allele has no effect on sporulation per se, and that the normal source of viable haploid spores is a small fraction of diploid nuclei ubiquitous in haploid plasmodia.

The acellular slime mold *Physarum polycephalum* proliferates in two alternative forms, plasmodium and myxamoeba. Plasmodia are multinucleate syncytia in which up to 10^8 nuclei divide with nearly perfect natural synchrony within a common cytoplasm. Under appropriate conditions of starvation and illumination, plasmodia may be induced to sporulate. When such spores germinate, they release uninucleate myxamoebae; clones of amoebae may be cultivated indefinitely on bacterial lawns. In heterothallic strains, the life cycle is completed when amoebae of different mating types fuse, leading to the formation of a new plasmodium (9). A single, highly polymorphic locus, *mt*, controls mating type (3, 6).

Colonia isolates differ from the heterothallic isolates in that plasmodia are formed within pure amoebal clones (17; H. A. von Stosch, M. van Zul-Pischinger, and G. Dersch, Abstr. Int. Bot. Congr., 10th, p. 481-482, 1964). This selfing ability has been mapped to the mating-type locus: strains carrying the *mt*h allele form plasmodia clonally. Plasmodia of *mt*h strains produce spores which, upon germination, release amoebae carrying the *mt*h allele. These spores germinate poorly. This defect appears to be a function of the *mt*h allele rather than of a separate genetic factor, since strains carrying heterothallic mating-type alleles in the Colonia background can produce spores with nearly 100% viability (1).

By genetic criteria, amoebae of both types of strain are usually haploid, whereas plasmodia of the heterothallic isolates are diploid. However, plasmodia of Colonia isolates have haploid deoxyribonucleic acid (DNA) levels (4). In the heterothallic isolates, the alternation of haploid and diploid states indicates that meiosis accompanies sporulation. This inference is supported by the observation that recessive alleles segregate in sporulation. The absence of a change in ploidy between plasmodia and amoebae of Colonia isolates might indicate that sporulation in these strains is not accompanied by a meiotic division. It was therefore proposed by Cooke and Dee (4) that "apogamic" might be a better term than "homothallic" to describe these strains.

In this paper, we explore an alternative possibility, that, in Colonia, amoebae are derived from meiotic spores. We study this by investigating the effect of nuclear diploidy on the viability of Colonia spores.

MATERIALS AND METHODS

Strains. All strains used in this study were derived from Colonia strain CL (4). Strain CL-2 is a diploid variant of CL isolated following a heat shock treatment (2). The derivation of CL-2 is described in Results.

Media. Liver infusion agar plates (0.05% liver infusion, 2% agar; 7) were used as the standard medium for amoebal culture and spore germination. Dilutions of spores and amoebae were made in an osmotically compatible phosphate-buffered salts solution (8) adjusted to pH 6.8. A semidefined axenic medium (N + C) (5) was used for growing plasmodia, and sporulation medium used for sporulation (5).

Culture methods. Amoebae were grown at 26°C on liver infusion agar plates covered with a lawn of *Escherichia coli*. Plasmodium-forming ability was scored on N + C (1:10 dilution) plates at 22°C. Mitotically synchronous macroplasmodia were prepared from axenic shake cultures of microplasmodia as described elsewhere (5, 13). Macroplasmodia generally underwent a round of synchronous mitosis at 8-h intervals beginning 4 h after plating microplasmodia. The time of mitosis was determined by phase-contrast observation of ethanol-fixed smears (10). Filters supporting plasmodia to be sporulated were transferred to sporulation medium, and melanized spores generally developed after 5 days of incubation at 26°C. Sporangia were allowed to mature an additional 5 days before spores were harvested and plated to measure viability.

Diploid plasmodia were prepared by the heat shock method of Brewer and Rusch (2). Filters supporting plasmodia were transferred to prewarmed 37°C plates for a 10-min interval immediately preceding metaphase of the second synchronous mitosis after plating. The success of the heat shock was monitored by examining ethanol-fixed smears 60 min later.

Nuclear isolation and DNA determination. Nuclei from plasmodia in G_2-phase were isolated by the method of Mohberg and Rusch (15) in nuclear homogenizing solution (0.24 M sucrose, 0.1% Triton X-100, 0.01 M $CaCl_2$, and 0.01 M tris(hydroxymethyl)-aminomethane-hydrochloride, pH 7.2).

Individual nuclei were assayed for their DNA content with a microfluorometric assay (16). Nuclei isolated by the above method were fixed in acetic acid-ethanol and air-dried along with control bull sperm on albumin-coated slides. The slides were then hydrolyzed for 10 min at 60°C in 1 N HCl and stained in a bisaminophenyloxdiazole solution (0.01% bisaminophenyloxdiazole, 0.1 M HCl, 0.5% $NaHSO_3$) for 2 h. Stained slides were then bleached in sulfite water (0.5% $NaHSO_3$, 0.5 M HCl), washed, and embedded in glycerol. Fluorometric measurements were made with a Zeiss photomicroscope, using ultraviolet epi-illumination through Zeiss UG-1 and BG-38 filters and photometric measurement through Zeiss barrier filters 41, 47, and 65. DNA determinations were based on arbitrary photometer units normalized to bull sperm controls on each slide (14). Although bull sperm values could vary considerably between slides (mean, 81.2; standard deviation, 80.3), there was little variation between the control nuclei within one slide (mean standard deviation, 6.1). Experimental values for each slide were normalized to a bull sperm value of 50 to allow comparison between slides; normalized values from replicate slides were reproducible (mean, 8.42; standard deviation, 1.18).

The ploidy series present within plasmodial nuclei provided a test of the linearity of the fluorometric assay: peaks with mean values of 6.5:14:26 fitted with an expected ratio of 1:2:4.

RESULTS

Plasmodia of strain CL sporulated readily when transferred to Daniel sporulation medium. Fully pigmented spores were generally produced within 5 days after transfer. Whereas the majority of spores appeared normal on microscopic inspection, only a small fraction (0.72%) formed amoebal clones on liver infusion agar plates (in contrast, 10 to 100% of the spores from diploid heterothallic plasmodia could germinate). One might suppose that the low viability of Colonia spores resulted from the failure to receive a full complement of genetic material. However, if the chromosome number of *Physarum* is 35 to 40 (14), and if unpaired chromatids segregate randomly, the maximum fraction of viable meiotic products would be only 2^{-35}. The observed viability far exceeded this value.

Viable spores might result from the presence of a fraction of diploid nuclei in the Colonia plasmodium. To test this, we assayed the DNA content of individual nuclei by microfluorometry (16).

In this method, a fluorochrome, bisaminophenyloxdiazole, is used to stain nuclei in a modified Feulgen reaction. Stained nuclei are excited by a beam of 365-nm light and fluoresce. Photometric measurements of the emitted visible light (500 to 600 nm) are proportional to DNA content (16). Figure 1 shows the distribution of values of nuclear DNA in CL. Indeed, a small fraction of CL nuclei in a "satellite" peak (P = 0.01 by Student's t test) at twice the haploid mean appeared to be diploid. Further support of the hypothesis that diploid plasmodial nuclei are the source of viable sporulation products was obtained by forming a diploid variant of CL. A heat shock immediately before metaphase can cause a plasmodium to skip mitosis and enter a second round of replication, leading to a doubling in nuclear DNA content (2). Such a treatment was used to construct CL-2, a homozygous diploid variant of CL. Most of the nuclei of CL-2 had nuclear DNA values at least twice that of CL (Fig. 1).

Parallel cultures of CL and CL-2 were sporulated, and the germination frequency was determined by platings on liver infusion agar plates. A comparison of spore germination frequencies with the fractions of the diploid nuclei isolated from the parent plasmodia is given in Table 1. The large increase in germination efficiency accompanying the increase in content of diploid nuclei in CL-2 is consistent with our hypothesis.

Heat shocking does not act by selecting a variant population. All amoebal clones emerging from CL-2 resemble Colonia by producing plasmodia at 22·but not at 30°C. Furthermore, plasmodia arising from these amoebae [CL2

(B)] match Colonia in nuclear DNA values (Fig. 1) and low spore viability (Table 1).

DISCUSSION

The germination efficiency of Colonia spores can be restored to near normal levels by heat shocking the parent plasmodium. This improvement in germination correlates approximately with the fraction of diploid nuclei in the sporulating plasmodium. The amoebae released are indistinguishable from Colonia amoebae, and the plasmodia they produce resemble Colonia in both nuclear DNA content and spore germination efficiency. Thus, the effect of heat shocking persists through vegetative plasmodial growth but is reversed by sporulation. Our hypothesis for explaining these observations is that the mechanism of sporulation in Colonia is the same as in heterothallic lines of *Physarum*, namely, meiosis.

In the case of Colonia, most haploid nuclei enter an abnormal meiotic division similar to that seen in haploid plants (12). There is an absence of bivalent associations, random distribution of univalents, and lagging chromosomes, which fail to group in a metaphase plate. Most mature spore nuclei have an abnormal, annular nucleolus (12).

If the low germination efficiency of Colonia spores is due to aneuploidy after pseudomeiosis of haploid nuclei, then abnormalities in Colonia spores should occur only after the time of meiosis. Spore cleavage and melanization precede meiosis in sporulating heterothallic plasmodia (11). Thus, spore melanization is not necessarily an indicator of the successful completion of meiosis. We propose that the observed cytological abnormalities and the inability of most spores to germinate are due to aneuploid nuclei produced by haploid pseudomeiosis. Several division schemes can be postulated to explain the occasional occurrence of functional spore nuclei. Alternatively, the rare diploid nuclei that we observed could be the source of viable meiotic progeny. Since the dip-

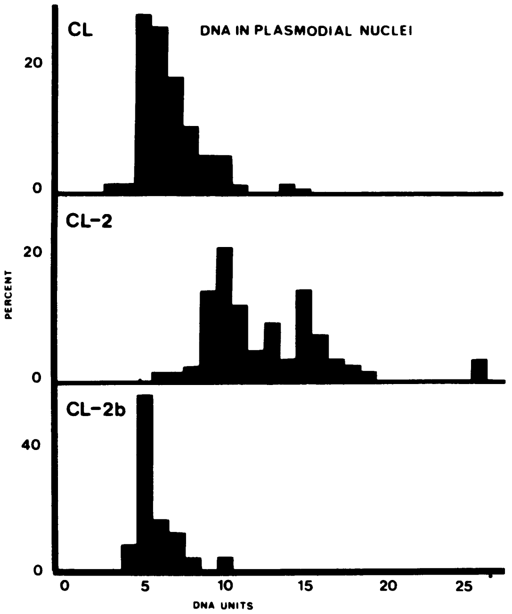

FIG. 1. *The distribution of DNA contents of individual nuclei. G_2 nuclei were isolated, and their DNA contents were determined fluorometrically as described in the text. Strains CL and CL-2 have been described in the text; strain CL-2(B) is a second-generation plasmodium derived from amoebae from CL-2 spores. Arbitrary photometer values, normalized to bull sperm controls, are plotted on the ordinate, and the frequency of nuclei with each value is plotted on the abscissa.*

TABLE 1. *Diploid nuclei content and spore germination*[a]

Strain	Nuclei[b] Haploid No.	Haploid %	Diploid No.	Diploid %	Germination (%)[c]
CL	123	98 ± 9	3	2.4 ± 1.3	0.72
CL-2	16	19 ± 5	69	81 ± 10	8.7
CL-2(B)	24	96 ± 20	1	4 ± 4	0.17

[a] Plasmodia prepared in parallel with those used for nuclear DNA measurements were sporulated. Spores were harvested 5 days after sporulation and plated on liver infusion agar plates at 26°C.

[b] The haploid and diploid fractions were calculated from the distributions shown in Fig. 1. The values from CL and CL-2(B) represent the totals under the respective peaks. Since there were overlapping distributions in the case of CL-2, all values within two standard deviations (standard deviation = 1.6) of the mean of the haploid CL peak (6.5) were considered haploid, and larger values (i.e., those greater than 9.7) were considered diploid. Thus, rare tetraploid nuclei were included in the diploid fraction.

[c] Percent germination is the percentage of spores that formed amoebal colonies after 10 days. A minimum of 200 colonies was counted to determine each value. Since spore germination in *Physarum* is quite variable, these data bear only qualitative significance.

loid nuclei were sufficient in number to explain the observed fraction of viable spores, they provide an adequate explanation for successful gametogenesis by haploid plasmodia.

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