PARASEXUAL GENETIC ANALYSIS OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM* A3

FRANK G. ROTHMAN AND ELLEN T. ALEXANDER

Division of Biological and Medical Sciences,
Brown University, Providence, Rhode Island 02912

Manuscript received August 2, 1974
Revised copy received April 2, 1975

ABSTRACT

Haploid strain A3 of the cellular slime mold *Dictyostelium discoideum* is valuable for biochemical studies because it is capable of axenic growth. Mutants of A3 temperature-sensitive for growth and resistant to the drugs cycloheximide, acriflavin, or methanol were isolated.——Heterozygous diploid recombinants, formed at low frequency by cell and nuclear fusion, were isolated by selecting temperature-resistant progeny of mixed cultures of two nonallelic temperature-sensitive haploids (LOOMIS 1969). Each drug-resistant mutation was found to be recessive. Two independently isolated methanol-resistant mutants were in one complementation group.——Diploids of A3 heterozygous for drug resistance formed drug-resistant segregants with a frequency of approximately 10^{-4}. Segregants selected for resistance to a single drug were either haploid or diploid; the fraction which was haploid varied from 0.11 to 0.86, depending on the selected marker. Segregants selected for resistance to two or three drugs were almost all haploid.——Using this parasexual cycle of diploid formation and haploidization, linkage of these temperature-sensitive and drug-resistance mutations to each other and to mutations studied by KATZ and SUSSMAN (1972) and by WILLIAMS, KESSIN and NEWELL (1974b) was analyzed. The methanol-resistant mutants were found to be partially resistant to acriflavin, and unlinked to the mutant selected for acriflavin resistance, which was methanol-sensitive. Of the expected seven linkage groups in *D. discoideum*, five, and a possible sixth, have been marked.——Linkage analysis of a mutant abnormal in morphogenesis showed that its phenotype results from two unlinked chromosomal mutations.

IN the life cycle of the cellular slime mold *Dictyostelium discoideum* both unicellular and multicellular stages are prominent. In the presence of food, solitary myxamoebae grow and divide. When deprived of food, they aggregate to form a multicellular organism containing about 10^5 cells. During the sixteen hours following aggregation, the multicellular aggregate undergoes morphogenesis to construct a sorocarp (fruiting body) which consists of a sorus of vegetative spores atop a thin stalk (Figure 1a). Thus, during the developmental phase, differentiation of the myxamoebae into two cell types, spore and stalk, takes place (RAPER 1940; BONNER 1967).

The accessibility of the unicellular stage and the ease of cloning permit ready isolation of mutants which are abnormal in morphogenesis. In some mutants, development is arrested at one of the normal morphogenetic stages; in others the

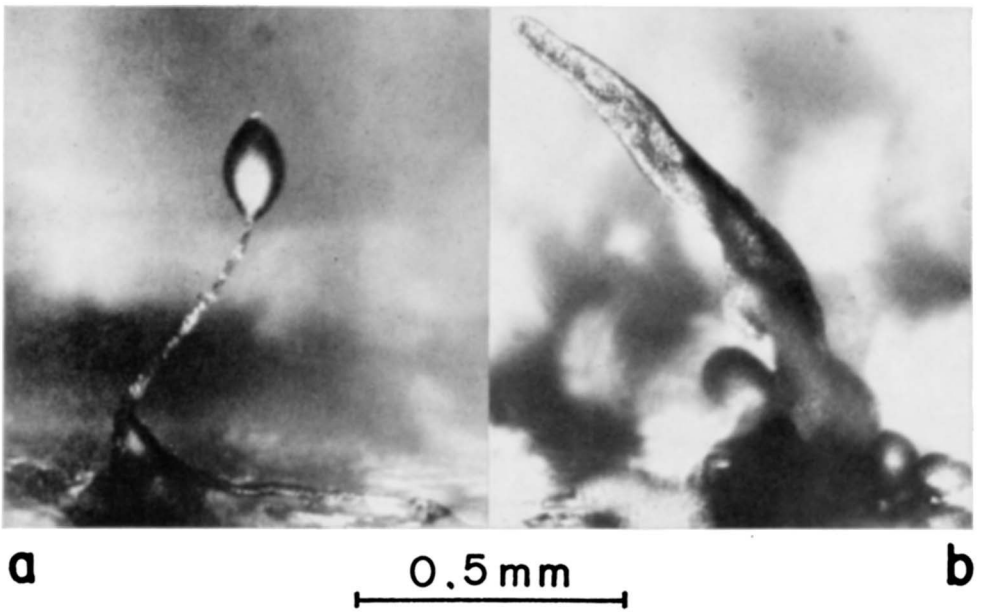

FIGURE 1.—Mature sorocarp of *D. discoideum A3*. b. "Gnarled finger" segregant of DR63 (genotype *devB1*) at terminal stage of morphogenesis.

morphology is aberrant (SUSSMAN 1966). Genetic analysis of morphogenetic mutants would be valuable in some investigations of the mechanisms of morphogenesis. A suitable method of genetic analysis was first introduced by KATZ and SUSSMAN (1972), who extended earlier work on a parasexual genetic system in *D. discoideum* (SUSSMAN and SUSSMAN 1963; LOOMIS and ASHWORTH 1968; SINHA and ASHWORTH 1969; LOOMIS 1969). Using pigment and spore color mutants, SUSSMAN and SUSSMAN (1963) demonstrated rare fusion of haploid cells from two strains to form heterozygous diploids, and the segregation of both parental and recombinant haploid progeny from these diploids. Since diploid formation and segregation each occur at low frequency, selective techniques in both steps are needed for routine genetic analysis. In the method of KATZ and SUSSMAN (1972), the haploid parental strains contain nonallelic mutations to temperature sensitivity for growth; one of the parents is also marked with a recessive drug-resistant mutation. The rare, heterozygous diploids formed by cell and nuclear fusion can be selected at a temperature which restricts growth of both haploid parents (LOOMIS 1969). Dominance relationships and complementation groups can be determined from the phenotypes of the diploids. The diploids obtained give rise to haploid segregants with a low frequency. The presence of the recessive drug-resistant marker permits selection of drug-resistant haploid progeny. The phenotype of these segregants with respect to unselected markers can then be scored to determine linkage groups. In this manner KATZ and SUSSMAN assigned five mutations in *D. discoideum* to three linkage groups.

Strain NC-4 of *D. discoideum* (RAPER 1940), used by KATZ and SUSSMAN, requires bacteria as food. For many biochemical experiments, strains which can grow axenically are of advantage and in some cases essential, to avoid interference by bacterial products. In this paper we describe parasexual genetic analysis in strain A3 (LOOMIS 1971) (also often called Ax3). This strain, derived from NC-4, is capable of axenic growth in a broth medium. In addition to two temperature-sensitive mutants we have isolated and studied mutants resistant to cycloheximide, acriflavin, or methanol, and a morphogenetic mutant deficient in aggregation.

Parasexual genetic analysis in *D. discoideum* A3 has also been reported by WILLIAMS, KESSIN, and NEWELL (1974a, b) who constructed heterozygous diploids from temperature-sensitive mutants of A3 and the NC-4 mutants of KATZ and SUSSMAN. We have determined the linkage relations between their mutants and ours. The combined data, in which five linkage groups and a possible sixth have been identified, are presented (Table 4). The haploid chromosome number is seven in A3 as well as in NC-4 (BRODY and WILLIAMS 1974).

KATZ and SUSSMAN (1972) noted that not all cycloheximide-resistant progeny of heterozygous diploids were haploid; some were diploids homozygous for cycloheximide resistance. Experiments described below indicate that homozygous methanol-resistant segregants can arise from mitotic crossing over. This conclusion has also been reached by WILLIAMS, KESSIN and NEWELL (1974b), by GINGOLD and ASHWORTH (1974), and by KATZ and KAO (1974).

We also present results which show that while selection for a single drug resistance yields both haploid and diploid segregants, simultaneous selection for two unlinked recessive markers yields almost exclusively haploids. Similar results have been reported in *Aspergillus nidulans* (KÄFER 1958; FORBES 1959).

MATERIALS AND METHODS

Media: SM broth (modified from SUSSMAN 1966) contained the following: glucose, 10 g; Difco bactopeptone, 10 g; Difco yeast extract, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; KH_2PO_4, 2.2 g; K_2HPO_4, 1 g; water, 1 liter. The pH was 6.3. SM agar was SM broth containing 15 or 20 g agar per liter. Drugs were sterilized by filtration and were added to the sterile molten agar to the following concentrations in mg per ml: acriflavin hydrochloride (Sigma), 0.1; cycloheximide (Sigma), 0.3. Methanol was added to 0.5% by volume. Plates and solutions containing acriflavin were handled in dim light. For axenic growth, HL-5 medium (COCUCCI and SUSSMAN 1970) was used. It contains: glucose, 15.4 g; Difco proteose peptone, 14.3 g; Difco yeast extract 7.1 g; $Na_2HPO_4 \cdot 7H_2O$ 0.96 g; KH_2PO_4, 0.49 g; water, 1 liter. Streptomycin sulfate was added to 0.5 mg per ml to cultures inoculated with spores from bacterial plates.

Methods of cultivation: D. discoideum was routinely grown on SM agar in association with *A. aerogenes* (SUSSMAN 1966). Stock plates of *A. aerogenes* were prepared on SM agar and incubated for at least 48 hours at 22°. For plating, slime mold spores or amoebae were premixed with *A. aerogenes* and 0.2 to 0.5 ml was spread per plate. For mass plates, 10^5–10^6 spores or amoebae were generally plated. Clonal plates were obtained by plating 100 or fewer cells per plate.

Liquid cultures of amoebae were grown in HL-5 in a gyrotory shaker bath at 110 r.p.m. 20 ml stock cultures were usually grown in 125 ml Erlenmeyer flasks and kept between 1×10^4 and 5×10^6 cells/ml.

Strains isolated as mutants or recombinants were cloned at least twice before a stock was prepared. Single clone isolation was usually achieved by streaking amoebae on a plate preseeded with bacteria.

Scoring phenotypes: All phenotypes were determined by spot tests on appropriate media. Depending on the particular circumstances, amoebae or spores from a purified clone were suspended in SM broth, and approximately 0.005 ml was spotted on plates seeded with bacteria. A grid of 12 spots on a 100 mm Petri dish was most convenient. Often two dilutions were spotted: one containing approximately 5×10^3 cells, which gave a confluent spot on a positive response, and one containing 50–100 cells, which gave countable miniplaques, permitting comparison of plating efficiency on various selective plates. Heterozygotes could often be recognized on drug-containing plates because resistant segregants gave rise to some clones in the more concentrated spot tests.

Selection of diploid cells: Temperature-resistant diploid strains were usually selected by the method of Loomis (1969), in which mixtures of the haploid, temperature-sensitive parental strains were plated on filters, harvested after 17 hours, disaggregated, and plated at the restricitve temperature. The method of Williams, Kessin and Newell (1974a), in which the parental mixtures were shaken in suspension for 15–17 hours and then disaggregated and plated, was also occasionally used. Temperature-resistant clones were typically obtained with a frequency of 10^{-6}. Many of these, but not all, were diploid. Some of the temperature-resistant haploid progeny had recombinant genotypes.

Measurement of spore volumes was performed according to the procedure of Sackin and Ashworth (1969): Mass plates were harvested on the sixth day after plating, when the fruiting bodies had just matured. Spores from older plates gave smaller volumes. Spore heads were picked with a sterile loop into filtered saline to a concentration of 10^5–10^6/ml (usually 50–100 heads into 50 ml). Spore volumes were determined on a Coulter Counter Model B with a 100 μm aperture tube, equipped with a particle size distribution plotter.

Cytological analysis: The ploidy of some strains was determined by Giemsa staining of the metaphase chromosomes (Brody and Williams 1974).

Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NG) (Aldrich Chemical Co.): Procedure 1 (modified from Fukui and Takeuchi 1971): Amoebae were grown in HL-5 to 1×10^6 cells/ml, centrifuged, and washed three times with M/60 Sorensen's phosphate buffer (pH 6.4). 1×10^8 washed cells were resuspended in 10 ml buffer containing 0.2 mg/ml NG and were shaken for 30 min at 22°. After centrifugation, the cells were washed three times with 10 ml buffer, resuspended in 10 ml buffer, shaken overnight at 22° and plated. Approximately 8% of the cells survived, and 20–25% of the survivors showed developmental abnormalities. Procedure 2 was the procedure of Yanagisawa, Loomis and Sussman (1967). The concentration of cells mutagenized was 1.4×10^7/ml. Survival was 1.2%, of which 6% were abnormal in development.

Mutants: Isolation and properties

Mutants temperature-sensitive for growth: Amoebae of A3 were mutagenized with NG according to Procedure 1 and plated clonally at 22°. Of 320 clones tested for growth at 27°, five did not grow. Two of these, HR4 and HR6, which were not "leaky" and had normal developmental morphology, were chosen for further use. Subsequent experiments showed that the optimal selective restrictive temperature for these strains was 26.3°. HR4, HR6, and derived strains carrying the same temperature-sensitive mutations revert to temperature resistance with a frequency of about 1×10^{-6}.

Cycloheximide-resistant mutants: Amoebae of HR4 were mutagenized with NG according to Procedure 2. The mutagenized culture was plated as mass plates on SM agar to allow phenotypic expression. Spores from the SM mass plates were plated on cycloheximide-containing plates, $1–3 \times 10^6$ per plate. Twelve cycloheximide-resistant clones were obtained from 2.7×10^7 spores plated. One of these, HR16, was used for the genetic studies.

Acriflavin-resistant-mutants: Amoebae of HR6 were harvested from cleared bacterial plates, mutagenized according to Procedure 1, and plated on SM acriflavin plates (6.6×10^4 cells per

plate). Of many resistant clones with normal morphology, one, HR7, was isolated for genetic studies.

Methanol-resistant mutants: Spontaneous mutants, resistant to 0.5% methanol, were obtained from strains HR7 and HR16 with a frequency of about 1×10^{-7}. Strain HR21 (from HR7) and strains HR22 and HR23 (from HR16) were purified and used in the genetic studies. HR23 is partially resistant to 1.25% methanol, but HR21 is sensitive at concentrations higher than 0.75%. The mutations to methanol resistance in HR22 and HR23 made these strains partially resistant to acriflavin. WILLIAMS, KESSIN and NEWELL (1974b) have isolated mutants resistant to 2% methanol which are cross-resistant to acriflavin.

The genotypes of all haploid strains of *D. discoideum* used in this study are listed in Table 1.

Morphological heterogeneity of some diploids: When diploids DR49a, DR49b, and three other diploids isolated from fusion of X9 and HR21 were plated clonally, heterogeneity in clone morphology was observed with respect to the spacing of fruiting bodies and the size of spore heads. A small fraction of the clones were aggregation-deficient. On replating, individual clones yielded progeny of several morphological types. The phenotyes of all clones, regardless of morphology, were those expected for these diploids: temperature-resistant, methanol-resistant, acriflavin-

TABLE 1

Genotypes of haploid strains of* D. discoideum

Name	Source	Mutant loci†						
		tsg	*cyc*	*acr*	*bwn*	*whi*	*axe*	*dev*
A3	M. SUSSMAN	+	+	+	+	+	A1,B1	+
HR4	NG‡ of A3	G4	+	+	+	+	A1,B1	+
HR6	NG of A3	F6	+	+	+	+	A1,B1	+
HR7	NG of HR6	F6	+	C4	+	+	A1,B1	+
HR16	NG of HR4	G4	A21	+	+	+	A1,B1	+
HR18	NG of HR7§	F6	+	C4	+	+	A1,B1	+
HR21	Spont. of HR7	F6	+	A21,C4	+	+	A1,B1	+
HR22	Spont. of HR16	G4	A21	A22	+	+	A1,B1	+
HR23	Spont. of HR16	G4	A21	A23	+	+	A1,B1	+
HR64	Seg. of DR57	G4	A21	A23	A1	+	A1,B1	+
HR65	NG of HR18	F6	+	C4	+	+	A1,B1	A1,B1
X2	R. KESSIN¶	A1	+	+	A1	+	A1,B1	+
X9	R. KESSIN¶	D12	A1	A1	A1	A1	+	+

All the diploid strains were derived from fusions of two haploids in this laboratory. Genotypes of the diploids are given in the text and other tables.

* Haploid strains isolated in this laboratory are designated with the prefix HR, and diploid strains with DR. Diploids isolated from fusion of the same two haploid strains are identified by the same number but distinguished by lower case letters, e.g., DR59a, DR59b.

† Genotype nomenclature for mutant loci is according to DEMEREC *et al.* (1966) and has been coordinated with that of WILLIAMS, KESSIN and NEWELL (1974b). Nonallelic loci with the same three-letter designation are assigned capital letters (e.g., *tsgF*, *tsgG*). Arabic isolation numbers denote different alleles. Results of the tests for allelism described in this paper are anticipated in the strain designations. Phenotypes: *tsgA*, *tsgD*, *tsgF*, *tsgG*, temperature-sensitive for growth; *cycA*, growth in the presence of 300 μg/ml cycloheximide; *acrA*, growth in the presence of 0.5% methanol and some growth in the presence of 100 μg/ml acriflavin hydrochloride; *acrC*, growth in the presence of 100 μg/ml acriflavin hydrochloride; *bwnA*, formation of brown pigment; *whiA*, white spore; *axeA*, *axeB*, growth in axenic medium when both are present; *devA*, *devB*, developmental mutant, see text.

‡ Abbreviations: NG, mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine; Spont., spontaneous mutant; Seg., segregant.

§ HR18 was selected as a mutant of HR7 resistant to 8-azaguanine. After cloning, the strain was no longer resistant to 8-azaguanine.

¶ WILLIAMS, KESSIN and NEWELL (1974a).

resistant, yellow, not brown, and heterozygous for cycloheximide resistance. The morphological heterogeneity of these diploids was not investigated further. DR51a, DR51b and ten other diploids from the fusion of X9 and HR23 did not show abnormal morphology, indicating that the peculiar behavior of DR49a and DR49b is not common to all diploids derived from axenic and nonaxenic parents.

Morphological heterogeneity of a different type, apparently due to segregation, was observed in DR59a, DR59b, and DR59c, diploids formed in fusions of X9 and HR18. When we examined about 40 clones of each strain we found that three-fourths of them had a normal spacing of sorocarps, with smaller sorocarps at the center. The other one-fourth of the clones contained widely spaced large sorocarps. Spot tests revealed that only the morphologically normal clones had the expected diploid phenotype: temperature-resistant, yellow, not brown, and heterozygous for resistance to cycloheximide, methanol, and acriflavin. Most clones with widely spaced sorocarps were no longer heterogygous for all markers tested.

RESULTS

Recessiveness of drug-resistant and temperature-sensitive mutations: Nine temperature-resistant strains were isolated from a fusion of HR16 (cycloheximide-resistant, temperature-sensitive) and HR21 (acriflavin-resistant, methanol-resistant, temperature-sensitive) using parental cells grown on bacteria. Four of the nine had spore volumes characteristic of diploids. Similar results were obtained when axenically grown parental cells were fused. When the diploids formed by these fusions were spotted on plates containing cycloheximide, acriflavin or methanol, a few resistant clones grew in spots where more than 1×10^3 cells had been deposited, indicating that the diploids were heterozygous for each drug-resistant marker, that each drug-resistant marker was recessive, and that drug-resistant segregants were formed at low frequency. One of the diploids, DR24, was chosen for detailed segregation studies. The average volume of spores of DR24 was 118 μm^3, while the volumes of the haploid parents were HR16, 72 μm^3 and HR21, 58 μm^3. The ploidy of these strains was also examined by cytological analysis (BRODY and WILLIAMS 1974). Mitotic figures with seven chromosomes were observed in HR16 and HR21. In DR24 we observed approximately 14 chromosomes, but the resolution was inadequate to distinguish between 13 and 14.

Formation of haploid and diploid drug-resistant progeny: KATZ and SUSSMAN (1972) reported that at least 10% of the cycloheximide-resistant segregants obtained from a heterozygous diploid were diploids homozygous for cycloheximide resistance. From heterozygous diploids of *A. nidulans*, selection for a single recessive marker also leads to both haploid and diploid segregants, but when two unlinked recessive markers are simultaneously selected, only haploid segregants are obtained (KÄFER 1958; FORBES 1959). At the suggestion of E. R. KATZ, we tested the effect of double-drug selection in *D. discoideum*. The results are shown in Table 2. In single-drug selection, the fraction of haploid segregants varied from 0.11 to 0.86, depending on the drug. Simultaneous selection for resistance to two or three drugs yielded 60 haploids and only 1 diploid.

In subsequent linkage analyses, haploid segregants were isolated either by selecting for resistance to two drugs or by selecting for one drug and choosing segregants having a recessive pigment or spore color phenotype.

TABLE 2

Segregation of drug-resistant progeny from strain DR24

Drugs in selective plate	Frequency of resistant segregants		Independent segregants scored for ploidy		
	Average number of resistant segregants per 10^4	Number of independent cultures analyzed	Total	Number haploid	Fraction haploid
Cycloheximide	2.3	54	65	7	0.11
Methanol	1.5	54*	85	73	0.86
Acriflavin	3.9	9	14	8	0.6
Cycloheximide + methanol	0.09	55	9	9	1.0
Methanol + acriflavin	1.7	25	8	8	1.0
Cycloheximide + acriflavin	1.0	24*	35	34	0.97
Cycloheximide + methanol + acriflavin	0.03	55	9	9	1.0

Mass plates were prepared from 85 clones of DR24. Duplicate plates containing 2×10^5 spores were plated from each culture tested on each type of plate. One, or in some instances two, segregants from each duplicate set were purified for spore volume and phenotype analysis. In the few cases where two segregants from the same set of selection plates were picked and were identical, only one was tabulated in order to record only the results of independent segregation events.

* Excludes one "jackpot", i.e., culture where early segregation resulted in a very high fraction of resistant progeny.

Linkage analysis of markers in DR24: The haploid segregants obtained from DR24 by selection for resistance to various combinations of drugs were scored for the phenotypes of unselected markers (Table 3). With selection for haploid segregants which carry one resistance marker, we expect 50% of the segregants to show the resistance determined by an unlinked gene, regardless of which haploid parent contributed it. A linked resistance gene should appear in all the selected segregants if it came from the same haploid parent as the selected gene, and in none of them if it came from the opposite haploid parent. Three-quarters of the haploid segregants should be temperature-sensitive if the two *tsg* genes are on different linkage groups and if neither is linked to the selected gene. All the segregants should be temperature-sensitive if the two *tsg* genes are linked or if the selected gene is linked to the *tsg* gene which came into the diploid in coupling with it. One-half of the segregants should be temperature-sensitive if the selected gene is unlinked to the *tsg* gene which came into the diploid in coupling but linked to the locus of the *tsg* gene from the other haploid parent. The following conclusions may be drawn:

1) Methanol resistance also confers acriflavin resistance. The data show that HR21 contains two unlinked mutations which confer resistance to acriflavin: one of these allows strong growth in the presence of the drug, the other only weak growth. The latter phenotype (RW) always (79 out of 79) segregates with methanol resistance (Table 3, lines 1b, 2a, 2c, 3c, 4a, 5a, 6b, 6c), consistent with the inference that the single mutation designated *acrA21* confers resistance to methanol and weak resistance to acriflavin. (The *acrC4* mutation confers strong resistance to acriflavin, but no resistance to methanol.) This inference is strength-

TABLE 3

Linkage analysis of cycA, acrA, acrC, tsgF *and* tsgG

Diploid DR24:	*cycA21* / +	+ / *acrA21*	+ / *tsgF6*	+ / *acrC4*	*tsgG4* / +	
	Phenotype					
Selected class	Cyc	Meth	Acr	Temp	Number	Line
Cycloheximide-resistant	(R)	S	S	S	4	1a
	(R)	R	RW	S	1	1b
	(R)	S	S	R	2	1c
					7	
Methanol-resistant	S	(R)	RW	S	32	2a
	S	(R)	R	S	17	2b
	R	(R)	RW	S	11	2c
	R	(R)	R	S	2	2d
	S	(R)	R	R	1	2e
					63	
Acriflavin-resistant	S	S	(R)	R	3	3a
	R	R	(R)	S	1	3b
	S	R	(RW)	S	1	3c
					5	
Cycloheximide- and methanol-resistant	(R)	(R)	RW	S	21	4a
	(R)	(R)	R	S	11*	4b
					32	
Cycloheximide- and acriflavin-resistant	(R)	R	(RW)	S	7	5a
	(R)	R	(R)	S	6	5b
	(R)	S	(R)	S	6	5c
	(R)	S	(R)	R	16	5d
	(R)	R	(R)	R	2	5e
					37	
Methanol- and acriflavin-resistant	R	(R)	(R)	S	5	6a
	R	(R)	(RW)	S	2	6b
	S	(R)	(RW)	S	4	6c
	S	(R)	(R)	S	1	6d
					12	
Cycloheximide-, methanol- and acriflavin-resistant	(R)	(R)	(R-RW)†	S	9	7a
					9	

Abbreviations: S, sensitive; R, resistant; RW, weakly resistant; Temp, temperature; Cyc, cycloheximide; Meth, methanol; Acr, acriflavin. Parentheses indicate selected markers.

* Five of these were intermediate between R and RW.

† Strength of response not scored.

ened by the finding that strains HR22 and HR23, which are spontaneous methanol-resistant mutants of an acriflavin-sensitive strain, are partially resistant to acriflavin. Cross resistance of methanol-resistant mutants to acriflavin has also been found by WILLIAMS, KESSIN and NEWELL (1974b). As shown below, all methanol-resistant mutants isolated by them and by us are in one complementation group. We shall retain the symbol *acrA* introduced by WILLIAMS, KESSIN and NEWELL (1974b) for this locus, although in our experiments the methanol-resistant phenotype was scored.

2) The *acrA21* and *acrC4* mutations are not linked: among the 34 selected strongly acriflavin-resistant segregants, 25 were methanol-sensitive (Table 3, lines 3a, b; 5b–e).

3) The *tsgF6* and *acrA21* mutations are linked. Of 63 independently arising segregants selected for methanol resistance, 62 were temperature-sensitive (Table 3, lines 2a–e). (The one temperature-resistant segregant is assumed to have resulted from mitotic crossing over before haploidization or from mutation.)

4) The *cycA21* mutation is not linked to the + allele of *acrA21*: among 44 cycloheximide-resistant segregants selected, only 28 were methanol-sensitive (Table 3, lines 1a–c; 5a–e).

5) The *tsgF* and *tsgG* loci are not on the same linkage group since if they were, all haploid segregants of DR24 would be temperature-sensitive. Among the 41 segregants for which methanol resistance (and the linked *tsgF6* mutation) was not selected, 23 were temperature-resistant (Table 3, lines 1a–c; 3a, b; 5b–e).

6) The *tsgG4* mutation is not linked to *cycA21*. The presence of *tsgG4* can be unambiguously scored in methanol-sensitive segregants, which carry the + allele of *tsgF6* by linkage. Of the 28 segregants selected for cycloheximide resistance that were methanol-sensitive, only 10 were temperature-sensitive (Table 3, lines 1a, c; 5c, d).

7) The *acrC4* mutation is not linked to the + allele of *tsgG4*: among 31 acriflavin-resistant segregants not carrying *tsgF6* (methanol-sensitive), only 21 were temperature-resistant (Table 3, lines 1a, c; 3a; 5c, d).

8) The relationship of *acrC4* to *cycA21*: among 39 segregants selected as resistant to cycloheximide, 11 were scored as having strong (or intermediate) resistance to acriflavin (Table 3, lines 1a–c; 4a, b). Among 10 selected strongly acriflavin-resistant segregants, 6 were cycloheximide-resistant (Table 3, lines 3a, b; 6a, d). These results suggest that *acrC* and *cycA* are on different linkage groups. Additional data on linkage of *acrC4* are presented below.

Linkage and complementation tests between the markers in DR24 and mutations isolated in other laboratories: The analysis of segregants of DR24 presented in the preceding section identified four independently assorting units, carrying *cycA21*, *acrA21-tsgF6*, *acrC4*, and *tsgG4* respectively. In this section we present results which correlate these four units with five linkage units described by KATZ and SUSSMAN (1972) and by WILLIAMS, KESSIN and NEWELL (1974b). The combined results are summarized in Table 4.

1) cycA21: The cycloheximide-resistant phenotype of DR51a and DR51b (Table 5) shows that our *cycA21* mutation is in the same complementation

TABLE 4

Linkage groups in D. discoideum

Linkage group: I	II	III	IV	V	VI (or V)
*acrB**	*acrA**‡	*acrC*‡	*bwnA*†	*tsgC**	*tsgG*‡
cycA†	*axeA**	*axeB**	*tsgB**		
devA‡	*devB*‡	*tsgA**			
spr†§	*tsgD*†				
tsgE†	*tsgF*‡				
	whiA†				

* Williams, Kessin and Newell (1974b).
† Katz and Sussman (1972).
‡ Results described in this paper.
§ Phenotype: round spore.

group as *cycA1*, and therefore on linkage group I. (The cycloheximide-sensitive phenotype of DR59a, DR59b, and DR59c confirms that *cycA1* is recessive.)

2) acrA21, acrA23, tsgF6: These mutations are assigned to linkage group II on the basis of two independent lines of evidence: (a) The methanol-resistant phenotype of DR49a and DR49b, and DR51a and DR51b (Table 5) indicates that *acrA21* and *acrA23* are in the same complementation group as *acrA1* and therefore on linkage group II. (The methanol-sensitive phenotype of DR59a, DR59b, and DR59c confirms that *acrA1* is recessive.) (b) All 70 haploid segregants of DR59b and DR59c (Table 6, all lines except 4c) were temperature-sensitive, indicating that *tsgF6* is on the same linkage group as *tsgD12*, namely II. The temperature resistance of the DR59 diploids shows, however, that *tsgD12* and *tsgF6* are not allelic.

3) acrC4: We assign *acrC4* to linkage group III on the basis of analysis of segregants of DR53, a diploid from HR7 and X2, which has the genotype

$$\frac{acrA21 \quad tsgF6}{+ \quad +} \qquad \frac{+}{tsgA1} \qquad \frac{acrC4}{+} \qquad \frac{+}{bwnA1}$$

TABLE 5

*Allelism of temperature sensitive and drug resistant mutants**

Diploids	Parents	Diploid genotype	Diploid phenotypes Cyc	Meth	Acr
DR49a,b	X9	$\frac{cycA1}{+}$ $\frac{acrA1 \; tsgD12 \; whiA1 \; +}{acrA21 \; + \; + \; tsgF6}$ $\frac{+}{acrC4}$ $\frac{bwnA1}{+}$	S	R	R
	HR21				
DR51a-k	X9	$\frac{cycA1}{+}$ $\frac{acrA1 \; tsgD12 \; whiA1}{acrA21 \; + \; +}$ $\frac{bwnA1}{tsgF6}$ $\frac{+}{tsgG4}$	R	R	RW
	HR23				
DR59a,b,c	X9	$\frac{cycA1}{+}$ $\frac{acrA1 \; tsgD12 \; whiA1 \; +}{+ \; + \; + \; tsgF6}$ $\frac{+}{acrC4}$ $\frac{bwnA1}{+}$	S	S	S
	HR18				

* Genotype abbreviations as in Table 1; phenotype abbreviations as in Table 3.

TABLE 6

Linkage studies of tsgF *and* acrC

Diploids DR59b,c				*cycA1* / +	*acrA1* / +	+ / *axeA*	*whiA1* / +	*tsgD12* / +	+ / *tsgF6*	+ / *acrC4*	+ / *axeB*	*bwnA1* / +	
		Selected class*					Phenotype					Segregants of	
Line	Cyc	Spore color	Pigment	Cyc	Temp	Meth	Acr†			DR59b	DR59c	Total	
1a	R	White	Brown	(R)	S	R	R			15	6	21	
1b				(R)	S	S	R			0	1	1	
2a	R	White	Not Brown	(R)	S	R	R			5	2	7	
2b				(R)	S	S	R			0	1	1	
3a	R	Yellow	Brown	(R)	S	S	S			19	6	25	
3b				(R)	S	S	R			0	1	1	
4a	R	Yellow	Not Brown	(R)	S	S	S			4	1	5	
4b				(R)	S	S	R			1	2	3	
4c				(R)	R	het	het			15	7	22	
5a	Axenic growth			S	S	S	R				6	6	

* Phenotype abbreviations as in Table 3; het: heterozygous.
† The presence of either *acrA1* or *acrC4* gives a strongly acriflavin-resistant phenotype, denoted as R.

Haploid segregants not carrying *tsgF6* were obtained by selecting for acriflavin resistance and screening for brown pigment and sensitivity to methanol. All 62 segregants of this phenotype, derived from at least 25 independent segregation events, were temperature-resistant, showing linkage between *acrC4* and the + allele of *tsgA1* which is on linkage group III. Care was taken to test initial segregant plaques of all sizes (including tiny ones) to avoid bias against slower-growing temperature-sensitive segregants.

The assignment of *acrC4* to linkage group III, linked to *axeB*, is supported by the acriflavin resistance of all six segregants of DR59c selected for axenic growth (Table 6, line 5a).

4) tsgG4: Our analysis of segregants of DR24 (Table 3) established that *tsgG4* is not on linkage groups I (unlinked to *cycA21*), II (not linked to *tsgF6*), or III (not linked to *acrC4*). Whether or not *tsgG4* is on linkage group IV was investigated by studying segregants of DR57, a diploid made from fusion of HR23 and X2. The results, shown in Table 7, indicate that *tsgG4* is not on linkage group IV

TABLE 7

Linkage analysis of tsgG

Linkage group	I	II	III	IV	
Diploid DR57:	*cycA21* / +	*acrA23* / +	+ / *tsgA1*	+ / *bwnA1*	*tsgG4* / +
Selected class				Temperature-Sensitive	Resistant
Cycloheximide- and methanol-resistant, brown				7	16
Cycloheximide- and methanol-resistant, not brown				12	11

since it segregates independently of *bwnA1*. These data also confirm that *tsgG4* is not on linkage group III since not all segregants of DR57 were temperature-sensitive.

Whether *tsgG4* is on linkage group V or on an as-yet-unmarked linkage group has not yet been determined. Strain NP7, which carries the only known marker on linkage group V, *tsgC7*, grows sufficiently at 26.3° to prevent selection of temperature-resistant diploids when it is fused with other temperature-sensitive strains derived from A3. [Earlier mapping of *tsgC7* was with a diploid selected at 27° from fusion of NP7 with an NC-4 derivative (KESSIN, WILLIAMS and NEWELL 1974).]

Preparation of a multiply marked tester strain: We have isolated developmental mutants in strains carrying *tsgF6* and *acrC4*. For linkage analysis of the developmental mutations we needed a multiply marked tester strain carrying a *tsg* mutation in a locus other than *tsgF*. The cycloheximide-resistant, methanol-resistant, brown, temperature-sensitive segregants of DR57 (Table 7) were suitable as tester strains, except that we did not know which *tsg* mutation(s) each segregant carried. To find out, we tried to fuse several segregants with X2 (*tsgA1*), and found one (HR64) which formed a temperature-resistant diploid indicating that it carried *tsgG4* but not *tsgA1*.

Linkage analysis of a developmental mutant: Strain HR65 was isolated as an aggregation-deficient mutant after NG mutagenesis of HR18 (*tsgF6*, *acrC4*). Most of the cells on mass plates of HR65 do not aggregate but remain as isolated amoebae. A very small, variable, fraction of the cells aggregate to form tiny, widely spaced, spherical mounds. We shall refer to this phenotype as "aggregateless".

HR65 was fused with tester strain HR64 and the temperature-resistant heterozygous diploid DR63 was isolated (genotype shown in Table 8). Spores of DR63 were plated on SM agar containing cycloheximide and methanol. Almost all of the clones which grew formed normal sorocarps, suggesting that the developmental mutation was on linkage group I or II, trans to *cycA21* or *acrA23*. We therefore examined haploid segregants obtained by selection for resistance to one drug. The results are presented in Table 8.

Among independently arising methanol-resistant segregants, 24 which formed normal sorocarps and 41 with non-normal phenotypes were isolated. Of the 24 normal ones, 23 were haploid as determined by spore volume. Among the 41 segregants with non-normal phenotypes, only one had the aggregateless phenotype of HR65 (Table 8, lines 2a-e). The other 40 formed sparsely spaced tiny or small sorocarps of normal shape. Many of the cells on mass plates of these segregants did not aggregate. We shall refer to this phenotype as "sparse tiny". As shown in Table 8, all 40 methanol-resistant sparse tiny segregants were cycloheximide-sensitive (lines 2a-d) and all 23 methanol-resistant segregants with normal sorocarps were cycloheximide-resistant (lines 1a-d). This indicates that the mutation responsible for the sparse tiny phenotype (*devA1*) is on linkage group I, *trans* to *cycA21* in DR63.

TABLE 8

Mapping of a developmental mutant

Diploid DR63:	*cycA21* / +	*acrA23* / +	+ / *tsgF6*	+ / *acrC4*	*bunA1* / +	*tsgG4* / +	+ / dev?	
				Phenotype*				
Line	Selected class	Cyc	Meth	Pigment	Temp.	Morphology		Number
1a	Methanol-resistant,	R	(R)	not brown	R	(normal)		4
1b	normal morphology	R	(R)	not brown	S	(normal)		1
1c		R	(R)	brown	R	(normal)		15
1d		R	(R)	brown	S	(normal)		3
								23
2a	Methanol-resistant	S	(R)	not brown	R	sparse, tiny		6
2b	mutant morphology	S	(R)	not brown	S	sparse, tiny		3
2c		S	(R)	brown	R	sparse, tiny		27
2d		S	(R)	brown	S	sparse, tiny		4
2e		S	(R)	not brown	R	aggregateless		1
								41
3a	Cycloheximide-	(R)	R	(brown)	R	normal		8
3b	resistant, brown	(R)	R	(brown)	S	normal		5
3c		(R)	S	(brown)	S	gnarled fingers		8
3d		(R)	R	(brown)	R	aggregateless		1
								22
4a	Acriflavin-resistant	R	R	brown	R	normal		1
4b		S	S	not brown	S	aggregateless		3
4c		R	S	brown	S	gnarled fingers		1
4d		R	S	not brown	S	large, stalkless		1
								6

* Abbreviations as in Table 3.

Among the cycloheximide-resistant segregants, only those making brown pigment were scored, in order to limit the analysis to haploids. Of 22 independently arising segregants, 13 formed normal fruiting bodies and 9 did not. The phenotype of 8 of the latter 9 differed from the phenotype of both HR65 and the sparse tiny methanol-resistant segregants. These 8 segregants formed gnarled fingerlike structures (Figure 1b). The ninth mutant segregant did not aggregate. As shown in Table 8, all 13 of the normal segregants were methanol-resistant (line 3a, b) while all 8 of the segregants forming gnarled fingers were methanol-sensitive (line 3c), indicating that the mutation leading to the gnarled finger phenotype (*devB1*) is on linkage group II, *trans* to *acrA21* in DR63.

These results suggested that the aggregateless phenotype of HR65 is the result of the expression of the two unlinked mutations *devA1* and *devB1*, each of which by itself gives rise to a different phenotype. The data obtained with segregants selected for acriflavin resistance (Table 8, lines 4a-d) strongly support this conclusion: of 24 independently arising acriflavin segregants, six were haploid. One of these had a unique morphological phenotype—large stalkless spheres—and

may have arisen by mutation. Among the other five haploids, the correspondence of phenotypes with respect to morphology and drug resistance was precisely what was expected: the three segregants which had the aggregateless phenotype like HR65 were all methanol- and cycloheximide-sensitive (line 4b); the one segregant which formed normal fruits was cycloheximide- and methanol-resistant (line 4a); and the one segregant which had the gnarled finger phenotype was cycloheximide-resistant, methanol-sensitive (line 4c).

Mitotic recombination: In *A. nidulans* most diploid segregants which are homozygous with respect to an originally heterozygous locus arise as a result of mitotic crossing over. Infrequently, diploid segregants arise following chromosome nondisjunction (FINCHAM and DAY 1971). The two mechanisms may be distinguished in cases where the parental diploid is heterozygous for two or more linked markers. Segregants arising from mitotic crossing over are heterozygous for markers located between the centromere and the exchange point, as well as for markers on the other arm of the chromosome undergoing exchange, but are homozygous for all markers located distal to the exchange point. Diploid segregants arising following nondisjunction of a chromosome will be homozygous for all markers on that chromosome.

Among eleven diploid methanol-resistant segregants of DR24 (genotype of DR24 shown in Table 3), two were temperature-resistant and nine temperature-sensitive. The two temperature-resistant ones cannot be homozygous diploid for *tsgF6*, which is linked to *acrA21*, and therefore could not have arisen from a nondisjunctional event. Since the spontaneous mutation frequency to methanol resistance is only about 10^{-7}, it is unlikely that the two (independent) temperature-resistant homozygous methanol-resistant diploids, which arose with a frequency of about 2×10^{-6}, were due to spontaneous mutation. They most probably are products of mitotic crossing over, suggesting that *tsgF6* is located between the centromere and *acrA21* or in the other arm. The occurrence of mitotic crossing over in *D. discoideum* has recently been reported (KATZ and KAO 1974; GINGOLD and ASHWORTH 1974; WILLIAMS, KESSIN and NEWELL 1974b).

DISCUSSION

KATZ and SUSSMAN (1972) reported that cycloheximide-resistant segregants of a heterozygous diploid strain of *D. discoideum* may be either haploid or diploid. We have found that the fraction of segregants which was haploid differed for three drug-resistance mutations we studied in DR24: 0.11 for cycloheximide, 0.6 for acriflavin, and 0.86 for methanol. By simultaneous selection for resistance to two drugs we obtained only haploid segregants (with one exception), at frequencies comparable to selection for a single-drug resistance (Table 2). Since diploid segregants arise (at least in part) from mitotic crossing over, we would expect the frequency of diploid segregant formation, and therefore the fraction of segregants which are haploid, to vary with the distance of the segregating marker from the centromere. From the data in Table 2 we calculate that haploidization and

mitotic crossing over in one chromosome occur with comparable frequencies of order of magnitude 10^{-4} to 10^{-5}. In double-drug selection, formation of a diploid homozygous for two unlinked resistant alleles requires two independent crossover events and therefore occurs at much lower frequency.

In the experiments described in this paper we analyzed only segregants of independent origin. Therefore, any unequal recovery of alleles was due either to preferential chromosome loss or to selection during growth prior to plating on selective plates. Analysis of the data in Tables 3, 6, and 8 reveals less than the expected 50% recovery of the following alleles: *cycA21*, 24 out of 92 (Table 3, lines 2a-e; 3a-c; 6a-d; Table 6, line 5a; Table 8, lines 4a-d); *acrC4*, 39 out of 138 (Table 3, lines 1a-c; 2a-e; 4a,b; Table 6, lines 1b; 2b; 3a,b; 4a,b); *bwnA*$^+$, 19 out of 70 (Table 8, lines 1a-d; 2a-e; 4a-d); *tsgG4*, 21 out of 105 (Table 3, lines 1a,b; 3a; 5c,d; Table 8, lines 1a-d; 2a-e). The fraction of temperature-sensitive segregants recovered was also low in the experiment reported in Table 7: 41% (19 out of 46) instead of the expected 75%. The low recovery of temperature-sensitive segregants may well be the result of slower growth due to the *tsg* genotype even at the permissive temperature. The recovery of only four *acrC4* segregants among 34 *acrA*$^+$ (methanol-sensitive) segregants of the nonaxenic/axenic hybrids DR59b and DR59c (Table 6, lines 3a, b; 4a, b) may be explained by the presence of the *axeA1* and *axeB1* mutations in these segregants due to linkage, resulting in slower growth on bacteria. However, since all our parental haploid strains had been mutagenized, some of the unequal recoveries of chromosomes may be due to undetected mutations. In particular, the very low simultaneous recovery of *acrC4* and *cycA21* in several experiments is not accounted for on the basis of known markers (Table 3, lines 1a-c; 3a-c; Table 6, line 5a.)

As shown in Table 4, mutations have been identified on five or six of the expected seven linkage groups of *D. discoideum*. Four mutations to cycloheximide resistance belong to a single complementation group, as do nine mutations to methanol resistance (Table 5 and WILLIAMS, KESSIN and NEWELL 1974b). Three unlinked loci conferring acriflavin resistance have been identified, one of them identical to the methanol-resistance locus (*acrA*). The *acrC4* mutation described in this paper provides a convenient selective marker on linkage group III. *AcrC4* can be used in conjunction with *acrA* mutations since it confers resistance only to acriflavin, not to methanol.

Our results with the aggregation-deficient mutant HR65 illustrate the application of parasexual genetic analysis to the study of developmental mutants. HR65, which was isolated after mutagenesis, was shown to contain two unlinked recessive mutations, each of which had a different phenotypic effect.

We thank M. V. TEDESCHI and M. GOYETTE for participating in some of the experiments, M. SUSSMAN, R. R. SUSSMAN, and E. R. KATZ for valuable advice, and R. H. KESSIN and K. L. WILLIAMS for communicating their results prior to publication. This work was supported by the National Science Foundation under grants GB-33461, GB-12614, and GB-43829X, and in part by grant PHS RR-07085-06 from the National Institutes of Health. S. ZIMMERING and R. H. KESSIN made valuable comments about the manuscript.

LITERATURE CITED

BONNER, J. T., 1967 *The Cellular Slime Molds.* Princeton University Press, Princeton.

BRODY, T. and K. L. WILLIAMS, 1974 Cytological analysis of the parasexual cycle in *Dictyostelium discoideum.* J. Gen. Microbiol. **82:** 371–383.

COCUCCI, S. M. and M. SUSSMAN, 1970 RNA in cytoplasmic and nuclear fractions of cellular slime mold amebas. J. Cell Biol. **45:** 399–407.

DEMEREC, M., E. A. ADELBERG, A. J. CLARK and P. E. HARTMAN, 1966 A proposal for a uniform nomenclature in bacterial genetics. Genetics **54:** 61–76.

FINCHAM, J. R. S. and P. R. DAY, 1971 pp. 115–127. In: *Fungal Genetics.* Third Edition. Blackwell, Oxford and Edinburgh.

FORBES, E., 1959 Use of mitotic segregation for assigning genes to linkage groups in *Aspergillus nidulans.* Heredity **13:** 67–80.

FUKUI, Y. and I. TAKEUCHI, 1971 Drug resistant mutants and appearance of heterozygotes in the cellular slime mould *Dictyostelium discoideum.* J. Gen. Microbiol. **67:** 307–317.

GINGOLD, E. B. and J. M. ASHWORTH, 1974 Evidence for mitotic crossing-over during the parasexual cycle of the cellular slime mould *Dictyostelium discoideum.* J. Gen. Microbiol. **84:** 70–78.

KÄFER, E., 1958 An 8-chromosome map of *Aspergillus nidulans.* Advan. Genet. **9:** 105–145.

KATZ, E. R. and V. KAO, 1974 Evidence for mitotic recombination in the cellular slime mold *Dictyostelium discoideum.* Proc. Natl. Acad. Sci. U.S. **71:** 4025–4026.

KATZ, E. R. and M. SUSSMAN, 1972 Parasexual recombination in *Dictyostelium discoideum*: Selection of stable diploid heterozygotes and stable haploid segregants. Proc. Natl. Acad. Sci. U.S. **69:** 495–498.

KESSIN, R. H., K. L. WILLIAMS and P. C. NEWELL, 1974 Linkage analysis in *Dictyostelium discoideum* using temperature-sensitive growth mutants selected with bromodeoxyuridine. J. Bacteriol, **119:** 776–783.

LOOMIS, W. F., JR., 1969 Temperature-sensitive mutants of *Dictyostelium discoideum.* J. Bacteriol, **99:** 65–69. ——, 1971 Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. Exptl. Cell Res. **64:** 484–486.

LOOMIS, W. F., JR. and J. M. ASHWORTH, 1968 Plaque-size mutants of the cellular slime mould *Dictyostelium discoideum.* J. Gen. Microbiol. **53:** 181–186.

PERKINS, D. D., 1962 Preservation of *Neurospora* stock cultures with anhydrous silica gel. Can. J. Microbiol. **8:** 591–594.

RAPER, K. B., 1940 Pseudoplasmodium formation and organization in *Dictyostelium discoideum.* J. Elisha Mitchell Sci. Soc. **56:** 241–282.

SACKIN, M. J. and J. M. ASHWORTH, 1969 An analysis of the distribution of volumes amongst spores of the cellular slime mould *Dictyostelium discoideum.* J. Gen. Microbiol. **59:** 275–284.

SINHA, U. and J. M. ASHWORTH, 1969 Evidence for the elements of a parasexual cycle in the cellular slime mould *Dictyostelium discoideum.* Proc. Roy., Soc. B **173:** 531–540.

SUSSMAN, M., 1966 Biochemical and genetic methods in the study of cellular slime mold development. pp. 397–409. In: *Methods in Cell Physiology*, Vol. 2. Edited by D. PRESCOTT. Academic Press, New York.

SUSSMAN, R. R. and M. SUSSMAN, 1963 Ploidal inheritance in the slime mould *Dictyostelium dicoideum*: haploidization and genetic segregation of diploid strains. J. Gen. Microbiol. **30:** 349–355.

WILLIAMS, K. L., R. H. KESSIN and P. C. NEWELL, 1974a Genetics of growth in axenic medium of the cellular slime mould *Dictyostelium discoideum*. *Nature* 247: 142–143. ——, 1974b Parasexual genetics in *Dictyostelium discoideum*: mitotic analysis of acriflavin resistance and growth in axenic medium. J. Gen. Microbiol. **84**: 59–69.

YANAGISAWA, K., W. F. LOOMIS, JR. and M. SUSSMAN, 1967 Developmental regulation of the enzyme UDP-galactose polysaccharide transferase. Exptl. Cell Res. **46**: 328–334.

Corresponding editor: D. R. Stadler