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Genetic analysis of actidione-resistant mutants in the Myxomycete *Physarum polycephalum*, Schw.

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1. INTRODUCTION

The Myxomycete (true slime mould) *Physarum polycephalum* is an organism ideally suited for the study of many biological problems including morphogenesis. However, although it is widely used as a tool by biochemists and biophysicists and precise methods exist for culturing the plasmodium on defined medium (Daniel & Baldwin, 1964), and for controlling mitotic synchrony (Guttes & Guttes, 1964) and sporulation (Daniel & Rusch, 1962), very little genetic analysis has been done (Dee, 1960, 1962). This deficiency is due chiefly to the difficulty of obtaining markers. No defined medium exists for the myxamoebae ('amoebae'); they are naturally resistant to most antibiotics and to many fungicides and many fruitless attempts have been made by the author to obtain resistance to several inhibitory drugs, including acriflavine and chloroquine.

The present paper reports the isolation and partial genetic analysis of several strains resistant to actidione (cycloheximide) an antibiotic produced by *Streptomyces griseus* (Whiffen *et al.*, 1946) which inhibits multiplication of *P. polycephalum* amoebae. Studies on the characteristics of these strains and the action of actidione-resistant alleles in plasmodia are also reported. The results of these studies show how the potentialities of *P. polycephalum* as a biological tool could be greatly increased by the further isolation of mutant strains and application of genetic analysis.

2. MATERIALS AND METHODS

(i) *Strains*

The strains of *Physarum polycephalum* used in this work have all originated from the strains of amoebae called *a* and *i* in previous papers. (Dee, 1960, 1962). These were previously said to carry mating types + and − respectively. Since two new mating types have recently been discovered, the terminology will in future be as follows: strains formerly called +, now called mt^1; strains formerly called −, now called mt^2. Further information on mating types will be published elsewhere.

(ii) *Culture method for amoebae*

Culture methods for amoebae are similar to those described previously (Dee, 1962) but with the following changes. A pure strain of *Escherichia coli* is now used as food for the amoebae in place of *Pseudomonas fluorescens*, which has been completely

eliminated from all cultures. Since *E. coli* grows poorly on unsupplemented water agar, Oxoid liver infusion is added. Basic medium for amoebae (SAL) is 20 g. agar, 0·5 g. liver infusion, 1 l. dist. water. Actidione-containing medium is made by adding actidione solution (sterilized by filtration) to melted SAL. A suspension of *E. coli* is spread on all medium used for amoebae. All strains were cloned before critical work by plating and selection from single plaques (for method, see Dee, 1962).

(iii) *Method of crossing*

A cross involves mixing together amoebae of different mating type, culturing the resulting plasmodium, inducing spore formation, plating spores to give plaques of amoebae and testing a sample of these on test plates. The following changes in methods described previously (Dee, 1962) have been made.

Plasmodium formation

Amoebae of the two strains are plated densely (approx. 10^5 total cells/plate) together with a suspension of *E. coli* on 2% water agar containing 0·0004 M *p*-aminobenzoic acid, a supplement which has been found to increase the frequency of plasmodium formation. After incubation for 6 or 7 days, yellow plasmodia are visible. A thin layer of well-cooled oat agar (autoclaved oats in 0·5% agar) is poured over each plate and the plasmodia grow through and cover this layer after a few more days' incubation.

Spore plating

The following method has been adapted from bacteriophage technique and consistently gives plaques from about 5% of spores plated. The spore suspension is plated on SAL at 200–400 spores/dish. One hour after plating, while the surface of the medium is still slightly wet, a thin layer of 'top agar' is added. Top agar (5 g. agar, 0·5 g. liver infusion, 1 l. water) is melted and dispensed into tubes (2·5 ml./tube) which are kept hot in a water bath at 46°C. until pouring. A few drops of a dense suspension of *E. coli* are added to each tube 1 or 2 min. before pouring and the contents of each tube are poured over one plate. As soon as the top agar has set, the plates are inverted and incubated. Plaques of amoebae appear after 7 or 8 days' incubation embedded in the soft top agar, and are easily sampled with a wire loop. It is assumed that each plaque arises from a separate spore. Since plaques take so long to appear, it is very unlikely that any spores hatch before the top agar is added.

Testing isolates

For all crosses described in this paper, mating type of progeny was tested on 2% water agar containing 0·0004 M *p*-aminobenzoic acid and the tester strains were *a* and *i*.

(iv) *Preservation of strains of amoebae*

The following simple method has been adopted from bacteriological technique. Amoebae are inoculated on SAL slopes and incubated for about 7 days, by which time most have encysted. Autoclaved liquid paraffin (B.P.) is then poured into each tube until the slope is completely covered and the tube securely plugged and stored at room temperature. When a growing culture is required, some amoebae are scraped off the slope by a platinum loop lowered through the paraffin. Transferred to fresh medium, these give an active culture in a few days. Strains have been successfully preserved by this method for more than a year.

3. ACTIDIONE-RESISTANT STRAINS

(i) *Isolation of strains resistant to* 4 μg./ml. *actidione*

Amoebae of one of the 'wild-type', sensitive strains, *a* or *i* were spread densely (approx. 10^6 cells/plate) on medium containing 4 μg./ml. actidione. There was slight multiplication, resulting in a sparse background layer, but plaques showed up clearly, because of their sharply-defined edge of heaped-up amoebae. Amoebae

Table 1. *Isolations from platings on* 4 μg./ml. *actidione*

Strain plated	No. plated		Resistant plaques		Resistant strains isolated
	Per dish	Total	No.	Frequency	
a	$0{\cdot}9 \times 10^6$	$16{\cdot}2 \times 10^6$	2	$1{\cdot}2 \times 10^{-7}$	*A7, A8*
i	$1{\cdot}1 \times 10^6$	$19{\cdot}8 \times 10^6$	2	$1{\cdot}0 \times 10^{-7}$	*A9, A10*

isolated from plaques gave rise to actidione-resistant strains (Table 1). Strains *A9* and *A10* showed poor growth on re-testing and are not discussed further. Strains *A7* and *A8* were cloned twice on 4 μg./ml. actidione and maintained on SAL slopes, and they remained resistant. Easy discrimination between these strains and the sensitive strains *a* and *i* was found in spot tests on 4 μg./ml. actidione. The range of discrimination is narrow: on 2 μg./ml., *a* and *i* grow; on 8 μg./ml., *A7* and *A8* fail to grow.

Since *A7* and *A8* were isolated from the same plating, they may both have arisen by the same mutational event. However, since both were used in the initial tests and crosses described below, strains which derive their resistance from *A7* will be said to carry act_7, and those deriving resistance from *A8* to carry act_8. The sensitive allele will be called *ACT*.

(ii) *Crosses between resistant and sensitive strains*

Each of the resistant strains *A7* and *A8* was crossed with the sensitive strain *i* and samples of the first generation progeny were tested for mating type and resistance to 4 μg./ml. actidione (Table 2). On actidione, the majority could be clearly classified

as resistant or sensitive but a few showed slight growth and were classified 'doubtful'. As usual, in the mating-type tests, some of the progeny failed to react with either tester strain and are classified 'no result', but the segregation $mt^1 : mt^2$ is 1 : 1 in the remainder. Segregation for actidione resistance vs. sensitivity is close to 1 : 1 and agrees with determination by a single pair of alleles. From the fully classified progeny, there is no evidence of linkage between the loci determining mating type and actidione resistance. The excess of recombinants in cross I may be caused by selective viability among the spores. The proportion of spores plated which gave rise to plaques was 7% in cross I (both samples) and 4% in cross II.

Table 2. *Crosses between resistant and sensitive strains*

	Strains	Supposed genotype
Cross I	*A7*	act_7 mt^1
	i	*ACT* mt^2
Cross II	*A8*	act_8 mt^1
	i	*ACT* mt^2

For cross I, results are given for two samples of the same batch of spores; 8 months elapsed between samples.

(*a*) *Segregation for actidione resistance*

Cross	Resistant	Sensitive	Doubtful	Total
I	42	53	1	96
	27	36	0	63
II	32	53	11	96

(*b*) *Segregation for mating type*

Cross	mt^1	mt^2	No result	Total
I	31	30	35	96
	31	23	9	63
II	35	33	28	96

(*c*) *Recombination among fully-classified progeny*

	Parental classes		Recombinant classes				
Cross	*act* mt^1	*ACT* mt^2	*act* mt^2	*ACT* mt^1	Total parental	Total recombinant	Total
I	9	12	17	22	21	39	60
	8	9	14	23	17	37	54
II	10	18	12	20	28	32	60

(iii) *Crosses between resistant strains*

To test whether *A7* and *A8* carried mutations at the same locus, two of their resistant progeny were crossed together (*A703*, *A802*, derived from crosses I and II respectively). A control cross of two resistant progeny of *A7* (*A703*, *A702*, derived from cross I) was also done. The results (Table 3) suggest that act_7 and act_8 are not at different loci, since the proportion of sensitive progeny in cross III ($act_7 \times act_8$) is no more than in cross IV ($act_7 \times act_7$). However, as stated above, *A7* and *A8* may have

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been derived from the same clone. All progeny of crosses III and IV were also tested for resistance to 8 μg./ml. actidione, but none were able to grow on this concentration. The proportion of plated spores which gave rise to plaques was 3% in cross III and 4% in cross IV.

Table 3. *Crosses between resistant strains*

	Strains	Genotypes
Cross III	*A703*	$act_7\ mt^1$
	A802	$act_8\ mt^2$
Cross IV	*A703*	$act_7\ mt^1$
	A702	$act_7\ mt^2$

(*a*) *Segregation for actidione resistance*

Cross	Resistant	Sensitive	Doubtful	Total
III	100	1	0	101
IV	90	1	10	101

(*b*) *Segregation for mating type*

Cross	mt^1	mt^2	No result	Total
III	35	29	37	101
IV	34	33	34	101

(iv) *Isolation of strains resistant to* 8 μg./*ml. and* 16 μg./*ml. actidione*

Strain *A701* ($act_7 mt^2$), resistant to 4 μg./ml. actidione, was plated at high density on plates containing 8 μg./ml. actidione and some clones stably resistant to this concentration were isolated. From one of these (*A16*), clones resistant to 16 μg./ml. were obtained in the same way. Table 4 shows the frequency with which these strains

Table 4. *Strains resistant to* 8 μg./*ml. and* 16 μg./*ml. actidione*

(*a*) *Platings to isolate strains*

Strain plated	Concentration of actidione (μg./ml.)	No. plated		Resistant plaques*		Resistant strains isolated
		Per dish	Total	Total no.	Frequency	
A701	8	$1{\cdot}2 \times 10^6$	1×10^7	30	3×10^{-6}	*A12–A17*
A16	16	$3{\cdot}0 \times 10^6$	3×10^7	30	1×10^{-6}	*A18–A24*

* On subsequent testing, about one-quarter of these plaques failed to show stable resistance.

(*b*) *Growth in spot tests*

\+ = growth − = no growth

Strain	Concentration of actidione (μg./ml.)		
	4	8	16
a	−	−	−
A701	+	−	−
A12–A17	+	+	−
A18–A24	+	+	+

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were isolated and their behaviour in spot-tests. The method of spot-testing is described in a previous paper (Dee, 1962) and consists essentially of inoculating a point on a plate with a large number of amoebae of a particular strain. Repeatable results are obtained by this method regardless, within fairly wide limits, of the age or number of amoebae inoculated. No genetic analysis has yet been done on these strains, but their existence suggests that the basis of actidione resistance may be multigenic in *Physarum polycephalum* as it is in some other organisms (Hsu, 1963; Wilkie & Lee, 1965).

(v) *Tests of plasmodia on actidione*

Repeated tests for resistance to actidione on both nutrient and non-nutrient medium were done with plasmodia heterozygous and homozygous for *act* alleles. Tests on non-nutrient medium were done by cutting pieces of plasmodium of

Table 5. *Tests of plasmodia on actidione*

(*a*) *Tests on SAL*

Expt.	Strain of plasmodium (mt^1)	Strain of plasmodium (mt^2)	Genotype	Area of plasmodium (sq. cm.)* SAL	SAL + 4 μg./ml. actidione	SAL + 8 μg./ml. actidione
(i)	*a* + *i*		*ACT*/*ACT*	50	2·8	1·5
	A7 + *i*		act_7/*ACT*	37	2·7	1·3
	A8 + *i*		act_8/*ACT*	46	2·2	2·0
(ii)	*a* + *i*		*ACT*/*ACT*	30	4·5	0
	A707 + *A702*		*ACT*/act_7	9	0	0
	A803 + *A805*		act_8/*ACT*	7	0	0
	A803 + *A702*		act_8/act_7	14	0·8	0

* Each figure is the average of three (for expt. i) or two (for expt. ii) plates of the same medium after 2 days' incubation.

(*b*) *Tests on oat agar* (*OA*)

Expt.	Strain of plasmodium (mt^1)	Strain of plasmodium (mt^2)	Genotype	Area of plasmodium (sq. cm.)* OA	OA + 4 μg./ml. actidione	OA + 8 μg./ml. actidione
(iii)	*a* + *i*		*ACT*/*ACT*	63	2·2	0·5
	A803 + *A805*		act_8/*ACT*	47	0·5	0
	A703 + *A702*		act_7/act_7	30	0·5	0·2
	A803 + *A702*		act_8/act_7	10	1·0	0

* Each figure is the average of two plates after 4 days' incubation.

standard size (approx. 0·5 sq. cm.) from oat agar cultures of the same age (2–3 days) and placing each piece at the centre of a 9 cm. diameter Petri dish containing SAL + actidione. At least two plates of each concentration were inoculated with each strain. The area of plasmodium was measured on each plate after 2 days' incubation;

and since areas on duplicate plates were always closely similar, they have been averaged (Table 5*a*). Plasmodium will not grow on unsupplemented SAL; its increase in area is due partly to growth as a result of feeding on the oat agar inoculum and partly to its thinning out as it spreads by protoplasmic flow.

For tests on nutrient medium, an attempt was made to control the inoculum size more precisely by allowing plasmodia to spread over SAL plates for 24 hours before cutting out pieces of standard size. Test medium was prepared by adding actidione and then oats to flasks of melted 0·5% agar. Areas of plasmodia were measured after 4 days' incubation and, as these were again closely similar for duplicate plates, they have been averaged (Table 5*b*).

In all these tests, there was little difference between the growth (or spreading) on actidione of plasmodia carrying one, two or no *act* alleles, and all were strongly inhibited by 4 μg./ml. actidione. Strains showed differences in their growth on drug-free medium, which may be caused by inherent or cultural differences between them but which do not seem to be correlated with genotype at the *ACT* locus. Since even plasmodia homogyzous for *act* do not show resistance to actidione, it seems that this allele, selected for its effect on amoebae, is not expressed in the plasmodium.

(vi) *Viabilities of resistant strains on actidione*

When amoebae are spot-inoculated on actidione (for method, see section 3, iv), a resistant strain forms a large plaque with a clearly-defined raised edge and a sensitive strain fails to multiply. When single cells of a resistant strain are plated

Table 6. *Viabilities of resistant strains plated on actidione*

Strain	Genotype	No. of plaques*		Percentage viability on actidione (plaques on actidione per 100 plaques on SAL)
		SAL	SAL + 4 μg./ml. actidione	
a	*ACT*	110·0	0	0
A7	act_7	51·8	32·2	62
A8	act_8	59·4	33·8	57
A701/S1†	act_7	107·3	70·3	66
A701/L1†	act_7	106·0	60·3	57

* Each figure is the average of four or five plates.

† *A701/S1* and *A701/L1* are strains isolated from *A701* which show stable clonal inheritance of small and large plaque size respectively. Further information on inheritance of plaque size will be published elsewhere.

out on the same concentration of actidione, however, plaques are formed by only 50–60% of the cells that form plaques on drug-free medium (Table 6). This result has been found for six different resistant strains tested, including *A16* on 8 μg./ml. actidione. The sensitive strain *a*, however, contains only about one cell per million capable of forming a plaque on 4 μg./ml. actidione (Table 1).

Since actidione resistance has been transmitted through sexual reproduction for

two generations and since resistance is maintained by clones through many subcultures on drug-free medium, its basis is presumably genic. Variation among the cells of a clone in their ability to form plaques on actidione may be caused by variation in the micro-environment or in the physiological state of the cells. Plaques formed by the resistant strains shown in Table 6 also appeared 4 days later on actidione than on SAL.

4. DISCUSSION

(i) *Actidione resistance*

Actidione has been shown to inhibit both protein synthesis and DNA synthesis in yeast (Kerridge, 1958) and mammalian cells (Bennett, Smithers & Ward, 1964). Cummins, Brewer & Rusch (1965) found that it inhibited protein synthesis and mitosis in *Physarum polycephalum* plasmodia. Several authors have attempted to identify its primary site of action (Siegel & Sisler, 1964; de Kloet, 1965).

Mutations to actidione resistance have been identified and mapped in *Saccharomyces cerevisiae* (Middlekauff *et al.*, 1957; Wilkie & Lee, 1965), *Neurospora crassa* (Hsu, 1963) and *Aspergillus nidulans* (Warr & Roper, 1965). Strains resistant to actidione have also been isolated in *Tetrahymena geleii* (Loefer & Matney, 1952) and the Myxomycete *Didymium nigripes* (Kerr, 1959). The genetic basis of resistance in *D. nigripes* cannot be easily studied because strains of amoebae cannot be crossed, the species being homothallic, but Kerr (1965) has studied segregation following the fusion of marked plasmodia.

It appears that a variety of organisms readily develop resistance to actidione, but little is known about the mechanism of resistance. Gundersen & Wadstein (1962) showed that when resistant cells of *S. pastorianus* were cultured in actidione-containing medium, the concentration of the drug was not decreased. Lee & Wilkie (1965) performed experiments with a radioactive analogue of actidione and concluded that resistant cells of *S. cerevisiae* were either taking in less actidione than sensitive cells or were inactivating the drug after its entry. In later experiments by Cooper and Wilkie (personal communication) evidence has been obtained that actidione actually enters the cells of both resistant and sensitive strains of this species. Whether this is true for *P. polycephalum* is not yet known.

Wilkie & Lee (1965) found that when resistant strains were grown in the presence of actidione, they had a pronounced lag phase followed by a period of normal growth and they concluded (Lee & Wilkie, 1965) that resistance develops in the presence of the drug. The resistant mutants of *P. polycephalum* also show an initial lag phase on actidione medium (section 3, vi). If one assumes that resistance develops during this phase (presumably by a process of enzyme induction or repression) it becomes easier to envisage why some cells of a resistant clone fail to grow when plated on actidione, since such a process is likely to be more sensitive to physiological or environmental factors than a 'built-in' resistance.

Development of resistance in the presence of actidione was also reported by Grover & Moore (1961) for two species of *Sclerotinia* and by Gundersen & Wadstein

(1962) for *Saccharomyces pastorianus*. These authors concluded that resistance was a temporary adaptation, since it was lost after a few subcultures on drug-free medium, but it seems possible that this loss might be accounted for by the selection of fast-growing back mutants, since the resistant strains were all slow-growing. Probable back mutation of this kind has been observed in *P. polycephalum* and will be reported elsewhere.

Kerridge (1958), with *S. carlsbergensis*, and Cooney & Bradley (1962), with cultures of *Tetrahymena* and human epithelial cells, found that cells were not killed by concentrations of actidione which inhibited multiplication. After prolonged exposure to the drug, they resumed normal growth immediately upon transfer to drug-free medium. The same result was found in the present work for amoebae of the sensitive strain *a* which resumed normal growth on SAL after 9 days' exposure to 4 μg./ml. actidione.

(ii) *Expression of genes in plasmodia*

The apparent lack of actidione resistance in plasmodia homozygous for *act* alleles, although disappointing in not allowing complementation tests, is interesting as an example of the expression of a particular gene in one developmental phase and not in another. There is insufficient knowledge of the mechanism of resistance to actidione to suggest a basis for the difference in gene expression in amoebae and plasmodium in this case, though an attractive hypothesis would be that it depends on a difference in the cell surface.

However, the phenomenon is interesting in drawing attention to the fact that *P. polycephalum* should be an ideal organism in which to detect 'switching on and off' of genes since all genes must pass through two different cellular environments in the normal life cycle. It should also be an ideal organism in which to study such effects, since both amoebae and plasmodium can be cultured indefinitely in their vegetative state, large quantities of both can be collected and accurate estimates of their multiplication or growth can be made.

SUMMARY

1. Improved methods for the culture and preservation of *Physarum polycephalum* amoebae have been developed.

2. Strains of amoebae have been isolated which are stably resistant to 4, 8 or 16 μg./ml. actidione.

3. Sensitivity vs. resistance to 4 μg./ml. actidione is probably controlled by a single pair of alleles at a locus unlinked to the mating-type locus.

4. Plasmodia homozygous or heterozygous for the allele determining resistance at this locus are no more resistant to actidione than plasmodia homozygous for the allele determining sensitivity.

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