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A gene conferring actidione resistance and abnormal morphology on *Physarum polycephalum* plasmodia

By JENNIFER DEE and RUSSELL T. M. POULTER
Department of Genetics, University of Leicester, England

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SUMMARY

Plasmodia of *P. polycephalum* homozygous and heterozygous for a mutation (*act*) which confers resistance to actidione on the haploid amoebal stage have been tested for resistance to actidione in liquid semidefined medium. Growth of homozygous resistant (*act*/*act*) plasmodia determined by protein or pigment assay was inhibited less by actidione than growth of homozygous-sensitive or heterozygous plasmodia. The mutant (*act*) is therefore recessive but, contrary to a previous report, confers resistance on homozygous plasmodia. The same mutation affects the morphology of homozygous (*act*/*act*) plasmodia on agar medium, causing slow growth, fragmentation and excessive sliminess.

1. INTRODUCTION

The isolation and genetic analysis of a mutation conferring resistance to actidione (cycloheximide) in the amoebal stage of the myxomycete *Physarum polycephalum* were reported by Dee (1966). Resistance was due to mutation at a locus (*act*) unlinked to the mating-type locus (*mt*). Plasmodia of *P. polycephalum* heterozygous (*act*/*ACT*) and homozygous (*act*/*act*) for the allele conferring resistance on amoebae were synthesized. Growth tests on agar-based nutrient and non-nutrient media in the presence and absence of actidione indicated (Dee, 1966) that these plasmodia did not show resistance to actidione greater than that of homozygous-sensitive strains (*ACT*/*ACT*). It was concluded that the actidione-resistant mutation selected in the haploid amoebae was not expressed in the diploid plasmodia, even when homozygous.

The present paper reports the results of more detailed studies on plasmodia of the same three genotypes. Growth of several strains of plasmodia of each genotype in shaken culture in liquid semi-defined medium in the presence and absence of actidione has been measured by means of pigment and/or protein estimation.

The results confirm that actidione resistance is recessive, but, unlike our previous studies, indicate that plasmodia homozygous for the resistant allele do show resistance to actidione. On agar medium, even in the absence of actidione, growth of homozygous-resistant plasmodia is poor and their morphology is characteristically abnormal. It is concluded that the actidione-resistant mutation selected in

amoebae is expressed in homozygous plasmodia conferring on them both resistance to actidione and abnormal morphology. Both effects are recessive in heterozygous plasmodia.

2. MATERIALS AND METHODS

(i) *Strains.* All strains used in the present investigation were derived from a cross between the actidione-resistant strain, A7 (genotype *act*, mt_1), originally isolated by Dee (1966) on agar medium containing 4 μg/ml actidione, and the sensitive strain, *i* (genotype *ACT*, mt_2). Resistant and sensitive progeny clones of amoebae of both mating types (mt_1 and mt_2) were isolated and crossed to give plasmodia of genotypes *act/act*, *act/ACT*, *ACT/ACT*. The progeny clones were assigned the numbers *A 7101–A 7150*. The plasmodia are numbered according to the amoebal clones used (e.g. *A 7124 + A 7120*), the clone of mt_1 in each case being written first.

(ii) *Culture of amoebae.* Culture methods for amoebae are similar to those reported previously (Dee, 1966), except that non-growing *Escherichia coli* is now used as food. This gives more repeatable results for tests of amoebae on drugs and widens the range of drugs that can be used. It is achieved by using a strain of *E. coli* carrying multiple nutritional requirements. The bacteria are grown in broth overnight, harvested by centrifugation and spread on 2% water agar on which they are incapable of growth. Amoebae are inoculated on the bacterial lawn. Using this method, the concentration of actidione necessary for discrimination between resistant and sensitive strains of amoebae is lower than that used previously with *E. coli* growing on liver infusion agar (3 μg/ml instead of 4 μg/ml).

Methods for crossing amoebae, testing them for resistance and plating spores were described previously (Dee, 1966).

(iii) *Culture of plasmodia.* A slight modification of the semi-defined medium published in Daniel & Baldwin (1964) was used ('SDM'). For convenience, the complete formula is given in Table 1. Except for a few tests on SDM-agar, plasmodia were grown and tested in liquid SDM in 150 ml conical flasks on a rotary shaker. Routine tests for contamination were made by inoculating samples in appropriate broths. Most plasmodia were synthesized from amoebae shortly before tests were made, but stored microsclerotia were sometimes used. These are produced by continued shaking of liquid cultures for some days after growth ceases and subsequent storage at 5 °C.

(iv) *Assays of plasmodial growth.* Growth of microplasmodia in liquid culture was assayed by determination of protein by the Biuret reaction and of pigment by the method described by Daniel & Baldwin (1964). Pigment from a washed, centrifuged sample of microplasmodia was extracted in 4% TCA-acetone (8 ml 100% (w/v) TCA; 92 ml acetone; 100 ml water) and the optical density determined at 400 mμ in a Gilford Spectrophotometer. The amount of pigment determined by this method has been shown to be proportional to protein by Daniel & Baldwin (1964). In the present study the relationship between assayed protein and pigment was investigated in the presence and absence of actidione.

Table 1

(*a*) Semi-defined medium (SDM)

Glucose	10 g	$FeCl_2.4H_2O$	0·06 g
Oxoid bacteriological peptone	10 g	$ZnSO_4.7H_2O$	0·034 g
Vitamin mix	100 ml.	Citric acid.H_2O	3·54 g
KH_2PO_4	2 g	Disodium EDTA	0·224 g
$CaCl_2$	0·9 g	Dist.H_2O up to 1 l.	
$MgSO_4.7H_2O$	0·6 g		

Adjust pH to 4·6 with 10 % NaOH. Add hematin solution immediately before use (1 ml/100 ml medium).

(*b*) *SDM agar:* Equal quantities SDM and melted 3 % agar mixed. Hematin solution (1 ml/100 ml) added after cooling to 40 °C.

(*c*) Vitamin mix

Inositol	119 mg	D-Pantothenic acid, Calcium salt	45 mg
Choline chloride	85·7 mg		
Biotin	158 mg	*p*-Aminobenzoic acid	8·16 mg
Thiamine HCl (Aneurin HCl)	424 mg	Folic acid	4·07 mg
		Vitamin B_{12} (Cyanocobalamin)	0·049 mg
Pyridoxal HCl	609 mg		
Pyridoxine HCl	87·2 mg	Riboflavin	43·6 mg
Niacin (nicotinic acid)	42·2 mg	Dist. H_2O	1 l.

(*d*) Hematin solution

1 % NaOH	100 ml	Hematin	0·05 g

Sterilized by autoclaving. Stored at 5 °C.

3. RESULTS

(i) *Relationship between pigment and protein*

Determinations of pigment and protein in a culture of one homozygous-sensitive (*ACT*/*ACT*) strain in the absence of actidione are shown in Fig. 1. Such cultures grow exponentially with a doubling time of about 10 h. After the first 24 h increase in pigment is directly proportional to increase in protein. Figure 2 shows that when actidione was added to a culture of the same strain after 25 h incubation, both protein and pigment production were strongly inhibited. Inhibition by actidione of protein synthesis in *P. polycephalum* plasmodia was reported by Cummins & Rusch (1966).

The high values for pigment during the first 24 h are due to the presence of haematin. This has been shown by pigment assays on dilution series of microplasmodia. When dilutions are made in SDM, the relationship between pigment optical density and microplasmodial concentration is linear for optical densities down to 0·1, but at greater dilutions no O.D. values lower than this are obtained. When SDM lacking haematin is used, the relationship is linear down to the lowest measurable values. Optical densities of 0·1 or less for pigment assays on dilute cultures in SDM are therefore not accurate estimations of microplasmodial content but are elevated because of the presence of haematin. Since the absorption

spectrum of haematin is close to that of pigment with a maximum near 400 mμ, it is impossible to differentiate between them. This effect of haematin was not mentioned by Daniel & Baldwin (1964), who showed protein and pigment to be proportional throughout the growth of a culture. In our experiments, the effect gave

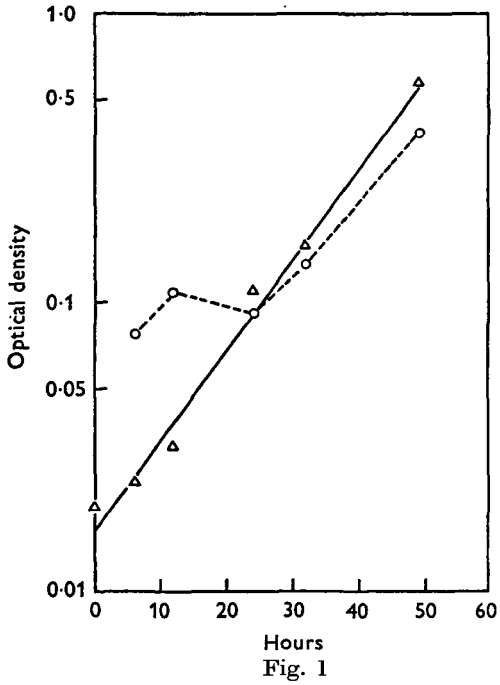


Fig. 1

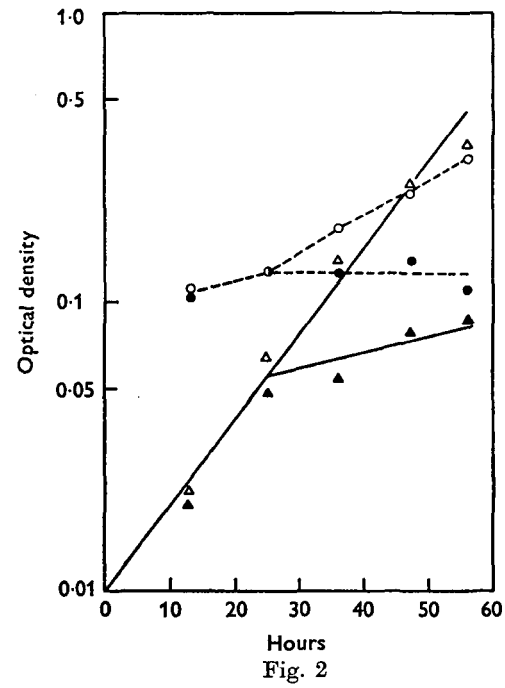


Fig. 2

Fig. 1. Protein and pigment increase in a liquid culture of microplasmodia of one homozygous sensitive (*ACT*/*ACT*) strain. Protein (△) plotted as O.D. at 550 mμ of the Biuret reaction. Pigment (○) plotted as O.D. at 400 mμ of extracted pigment.

Fig. 2. Effect of adding actidione to a culture of a homozygous sensitive (*ACT*/*ACT*) plasmodial strain. △, Protein and, ○, pigment for control flask. ▲, Protein and, ●, pigment for flask to which 1 μg/ml actidione was added at 25 h. For other details see Fig. 1.

incorrect optical density values for pigment in the first 24 h of growth only. This was not a source of error in growth tests since estimations were made after 2 or 3 days.

(ii) *Growth tests of different genotypes in liquid culture*

In tests performed to compare the growth of strains of different genotype in the presence and absence of actidione, the basic method was as follows. A 'starter' culture of each strain in liquid SDM was incubated for 24 h and used to inoculate four experimental flasks, two of them containing 1 μg/ml actidione in SDM and the other two SDM alone. A 24 h 'starter' culture was used to ensure that the plasmodia were in the log. phase of growth at the beginning of the experiment. A large inoculum (5 ml of starter culture added to 45 ml medium) was used because preliminary experiments using smaller inocula gave more variable results. Preliminary experiments using different concentrations of actidione showed that

1 μg/ml was the most suitable for discriminating between strains. The flasks were incubated for either 2 or 3 days (see below) and then sampled for protein and/or pigment estimations. Values obtained for duplicate flasks were always closely similar and were averaged. The average optical density value obtained for cultures in SDM + actidione was then expressed as a percentage of the value for SDM alone, to give a measure of resistance to actidione. Two series of growth tests were performed: one in which protein estimations were done and another in which only pigment was estimated.

Table 2

Strain	Genotype	Expt	Protein (mg/ml) SDM (*a*)*	Protein (mg/ml) SDM + *act* (*b*)*	% age growth in actidione (*b*/*a* %)
A 7119 + A 7120	*ACT/ACT*	I	0·576	0·036	6·25
		II	1·320	0·228	17·27
		III	1·254	0·138	11·00
A 7119 + A 7103	*ACT/act*	I	0·327	0·084	25·69
		II	0·846	0·192	22·69
		III	0·798	0·147	18·42
A 7119 + A 7137	*ACT/act*	III	1·194	0·123	10·30
A 7140 + A 7118	*act/act*	I	0·297	0·147	49·49
		II	0·348	0·144	41·38
		III	0·558	0·219	39·24

*Average for two flasks of same medium.

(iii) *Growth tests using protein estimation*

Results of three experiments in which protein was estimated are shown in Table 2. Samples were taken after 2 days.

(iv) *Growth tests using pigment estimation*

Seven experiments were done in which only pigment was estimated. Samples were taken after 3 days. In each experiment, strains of different genotype were compared, the total number of strains involved being five *ACT/ACT*, six *ACT/act* and five *act/act*. Results are summarized in Fig. 3. They show that resistance to actidione was not significantly different in *ACT/ACT* and *ACT/act* but was greater in *act/act* plasmodia.

(v) *Results on agar medium*

Two *ACT/ACT* and two *act/act* strains were inoculated on plates of SDM agar containing 0, 1, 2 and 3 μg/ml actidione. Both *ACT/ACT* strains showed the same amount of growth on 0, 1 and 2 μg/ml actidione but were partially inhibited by 3 μg/ml. The *act/act* plasmodia grew very poorly on all plates, including the controls, on which they had reached a size of only a few square centimetres after 3 days incubation, when the *ACT/ACT* plasmodia had extended to the edges of the plates. The morphology of the *act/act* plasmodia was characteristically abnormal, even in

the absence of actidione; the plasmodia becoming fragmented into many pieces which migrated over the plates, increasing in area only slowly and leaving very slimy tracks (Plate 1). A closely similar morphology was later found in all *act/act* strains and it has not been observed in plasmodia of other genotype except as a stage in senescence (R. T. M. Poulter, unpublished).

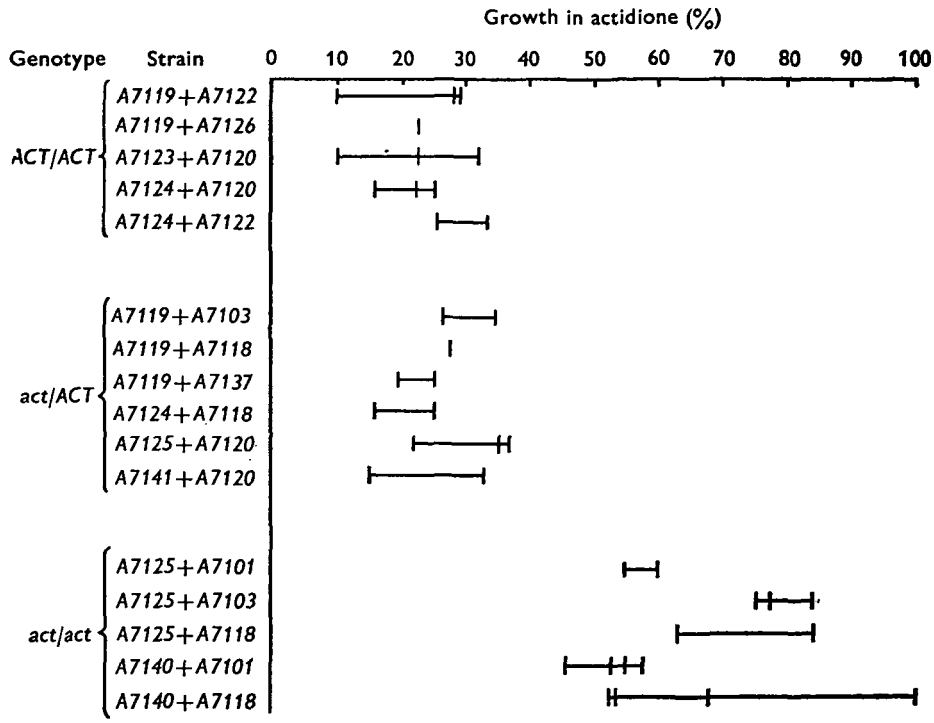
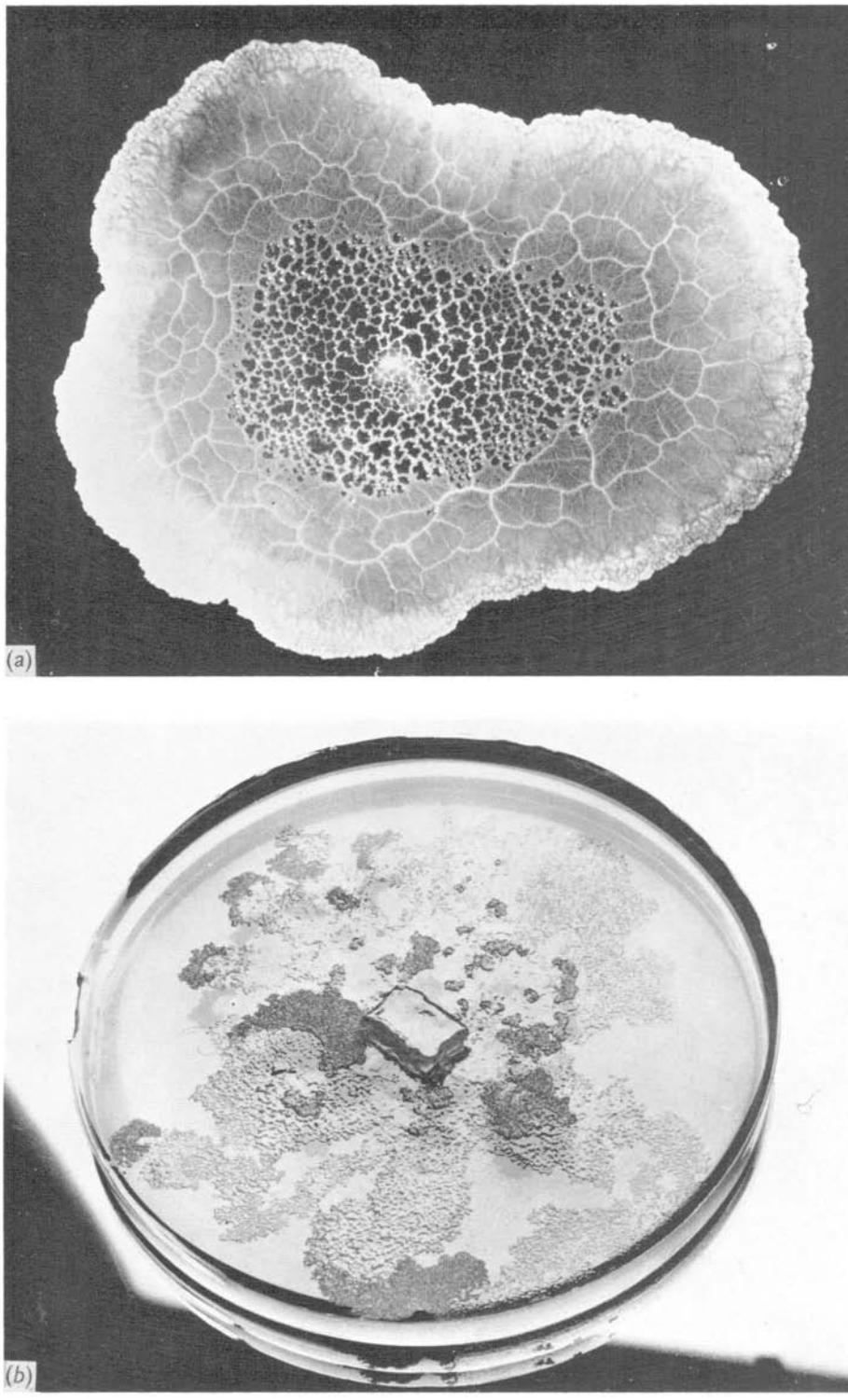


Fig. 3. Results of growth tests in which only pigment assays were made. Scale shows percentage growth in acitidione, i.e. pigment O.D. in SDM + actidione flasks as a percentage of pigment O.D. in SDM flasks. Each vertical line represents the result for one strain in one experiment; horizontal line shows range of results for the same strain.

4. DISCUSSION

The experiments on plasmodia in liquid semi-defined medium show that growth of homozygous actidione-resistant plasmodia (*act/act*), measured in terms of assayed protein or pigment, is inhibited less by actidione than is growth of homozygous sensitive (*ACT/ACT*) or heterozygous plasmodia. Thus the actidione-resistant mutation isolated in amoebae is recessive but confers resistance on homozygous plasmodia. The mutation has the additional effect, also recessive, of conferring abnormal morphology on plasmodia grown on agar in the presence or absence of actidione. These plasmodia grow so poorly that no clear resistance to actidione is shown in agar culture. This effect accounts for the failure of previous work (Dee, 1966) to detect resistance in homozygous plasmodia.



(*a*) A plasmodium of normal morphology growing on SDM agar. The plasmodium has spread out evenly from the point of inoculation. (*b*) A homozygous actidione-resistant (*act*/*act*) plasmodium on SDM agar. The agar block at the centre carried the inoculum. The plasmodium (dark areas) has fragmented into many pieces, which are migrating around the plate leaving very slimy tracks.

In yeast, both recessive and semi-dominant mutants resistant to actidione have been studied and experiments using cell-free systems (Cooper, Banthorpe & Wilkie, 1967) have shown that the resistance of recessive mutants resides in their ribosomes. Recessive resistance in *P. polycephalum* may also be a characteristic of the ribosomes. If this is so, the abnormal plasmodial morphology might be caused by malfunctioning of ribosomes in the absence of the drug; for example, by an increased frequency of misreading of messenger RNA. It is known that some drug-resistant mutations in bacteria which involve changes in the ribosomes can alter the detailed translation of messenger RNA. (For review, see Weisblum & Davies, 1968.)

This is the first example in *P. polycephalum* of a mutation selected in amoebae being found to have a characteristic effect on plasmodial morphology.

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