The Genetic Basis of Actidione Resistance in *Neurospora*

By K. S. HSU

*Department of Biological Sciences, Stanford University,
Stanford, California, U.S.A.*

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SUMMARY

Seven actidione-resistant mutants recovered from separate strains of *Neurospora crassa* are due to mutations of single genes at either of two loci: *act-1* (located between *nit-1* and *aur* in linkage group I) and *act-2* (located close to *sp* in linkage group V). Mutant alleles at both loci are dominant over their wild-type alleles in heterokaryons. Both genes are located in well marked chromosome arms, and scoring is distinct.

INTRODUCTION

Antibiotic-resistant mutants have been used routinely in bacterial genetics since Demerec (1945) demonstrated the mutational origin of penicillin resistance in *Staphylococcus aureus*. However, no such mutants have been reported in *Neurospora crassa*. The usefulness of drug-resistant mutants for genetic studies in such fungi as *Aspergillus nidulans* (Roper & Käfer, 1957), and the recent finding that *Neurospora* mutants resistant to the acridine dye acriflavine are controlled by chromosomal genes which can be used as markers, initiated the attempt to test several categories of drugs, including a number of antifungal antibiotics, with the aim of recovering useful markers. Mutants resistant to actidione have been found. The genetic basis of resistance and sensitivity to this antibiotic is presented in this paper. (A preliminary account of some of these results has been given [Hsu, 1962, and *Neurospora Newsletter*, **1**, 5].) Actidione resistance due to genic difference has previously been reported in *Saccharomyces cerevisiae* (Middlekauff *et al.* 1957) and it was on certain yeasts that actidione was first shown to have high toxicity (Whiffen, 1948).

METHODS

Throughout the experiments, media containing actidione (Upjohn) were sterilized by autoclaving for 10 min. at 121°. Conidiating colonial strains were selected for use as the starting strains. They were either double mutants or triple mutants, all in the background of St Lawrence stocks 74A and 73a. The genotypes of these strains and the isolation numbers of the alleles involved are listed in Table 1. The wild-type strains used in the present study were STA4, a vegetative reisolate of 74A, and Pa, derived from St Lawrence stocks by Perkins (1959).

For isolation of mutants, conidia from the sensitive strains were spread on the surface of medium N (Vogel, H. J., *Microb. Genet. Bull.* **13**, 42) containing 10 µg. actidione/ml., which was supplemented with 1% sucrose and solidified by 1.5% Difco agar. The actidione concentration was the lowest at which no growth was observable when 10⁶ to 10⁷ conidia from the sensitive strains were spread on plates

of graded concentrations of the antibiotic. The plated conidia were exposed to ultra-violet irradiation giving 50–75 % killing. Resistant colonies were selected after 5 or 6 days at 25°. Whenever more than one colony was found on the plates receiving conidia from the same strain, only one colony was selected to ensure that the mutants obtained had arisen from separate mutational events.

Each putative mutant was progeny tested by crossing with a standard wild-type strain. Ascospores were collected as unordered tetrads (Strickland, 1960) in order to detect whether the mutant strain was homokaryotic for the mutant allele.

The techniques used for crossing, for isolating random spores, and for linkage detection were similar to those used by Perkins (1959). Dry-weight assays (Ryan, 1950) were employed with modifications to measure quantitatively the sensitivity of different homokaryotic and heterokaryotic strains to this antibiotic. Conidial suspensions were inoculated into 125 ml. Erlenmeyer flasks containing 20 ml. of medium N with or without actidione. Dry weights were measured after four days at 25°.

RESULTS

Position of loci. Seven actidione-resistant strains of independent origin were isolated and subjected to transmission tests. In each case, a 2:2 segregation for resistance and sensitivity was demonstrated in individual unordered tetrads obtained from a mutant × wild-type cross, where two or three known, visible markers were also segregating. It appeared in each case that resistance had arisen through the mutation of a single Mendelian factor. Subsequent tests assigned the seven mutants to two independent loci: *act-1* and *act-2*. The assignment was based on the localization of the allele KH 52 in linkage group I and KH 53 in group V, and by subsequent tests of the remaining mutants with these two for allelism. Two mutants were tentatively considered to be allelic, if no wild type was observed within a small population taken from the progeny of a mutant × mutant cross. (The population sizes were 37, 62, 40, 46 and 29 of the crosses KH 51 × KH 52, KH 54 × KH 52, KH 56 × KH 53, KH 57 × KH 52, and KH 58 × KH 52, respectively.) Table 1 gives the locus to which each mutant was assigned, and the original strain from which each mutant was isolated.

Table 1. *Loci and strains of origin of actidione-resistant mutants*

Isolation no.	Locus	Strain of origin
KH 51	<i>act-1</i>	<i>cr(B123): bal(B56).A</i>
KH 52	<i>act-1</i>	<i>cr(B123): cot(C102).A</i>
KH 53	<i>act-2</i>	<i>cr^L: cot(C102): ylo(Y30539y).A</i>
KH 54	<i>act-1</i>	<i>cr(B123): bal(B56).a</i>
KH 56	<i>act-2</i>	<i>cr(B123): ylo(Y30539y).a</i>
KH 57	<i>act-1</i>	<i>cr^L, nit-1(34547), aur(34508).a</i>
KH 58	<i>act-1</i>	<i>cr(B123), rg(B53).a</i>

Linkage data. Linkage data of *act-1* and *act-2* based on three-point crosses are given in Table 2. The data indicated that the gene *act-1* is located between *nit-1* (nitrate-1) and *aur* (aurescent) in the right arm of linkage group I, and that *act-2* is close to *sp* (spray) in the right arm of linkage group V.

The double mutant. The double mutant was synthesized by crossing *act-1* (KH 52) × *act-2* (KH 53). A 3:1 ratio for the resistant and sensitive phenotypes was

observed in the progeny of such a cross, as expected on the basis of independent assortment of genes at these two loci. Among the resistant segregants, one-third were apparently slower in growth in the minimal slants as compared with the

Table 2. *Three-point data on random segregants from crosses involving act-1 and act-2*

Zygote genotype and recombination percentage	Parental combinations	Recombinations			Total and percentage germination	Marker isolation number
		Singles, region 1	Singles, region 2	Doubles, regions 1 and 2		
$\frac{+ \quad \text{act-1} \quad +}{cr \quad + \quad aur}$ 17.5 17.5	24 21	3 4	5 2	3 1	63 63 %	B 123 KH 52 34508
$\frac{+ \quad \text{act-1} \quad +}{nit-1 \quad + \quad aur}$ 6.3 4.7	33 24	2 2	1 2	0 0	64 64 %	34547 KH 52 34508
$\frac{+ \quad \text{act-2} \quad +}{lys-1 \quad + \quad inos}$ 25.7 14.3	39 47	15 19	9 9	1 1	140 72 %	33933 KH 53 37401
$\frac{+ \quad sp \quad inos}{act-2 \quad + \quad +}$ 3.1 6.2	42 47	1 1	3 2	1 0	97 32 %	KH 53 B 182 37401

Numbers of progeny are given in the body of the table. The numbers in the top row of each cross represent the genotypes which contain the plus allele of the left-most marker. Regions are numbered from left to right, and isolation numbers in the last column are listed in the same order. In the last cross the order of *act-2* and *sp* is arbitrary. For descriptions of markers, see Perkins (1959) and Barratt, Newmeyer, Perkins & Garnjobst (1954).

Table 3. *Test crosses identifying double mutants from the cross act-1 (KH 52) $\times$ act-2 (KH 53)*

Cross	Type of cross	Progeny class*		
		A	B	C
1	Normal-growing, resistant $\times$ sensitive	28	0	22
2		27	0	28
3		25	0	37
4		34	0	30
5		26	0	32
6		23	0	25
7		38	0	32
8	Slow-growing, resistant $\times$ sensitive	35	17	14
9		33	24	11
10		26	16	10

* A = Resistant to actidione (10 $\mu\text{g./ml.}$), normal growth in the minimal slants.

B = Resistant to actidione (10 $\mu\text{g./ml.}$), slow growth in the minimal slants.

C = Sensitive to actidione (10 $\mu\text{g./ml.}$), normal growth in the minimal slants.

remaining two-thirds, and with the sensitive segregants. In order to separate the double mutant from the single mutants, seven normal-growing, resistant types and three slow-growing, resistant types were backcrossed to the wild types (Table 3). While the former type of crosses gave 1:1 segregation for resistance and sensitivity and no slow-growing segregants, the latter type gave 3:1 segregation for resistance

and sensitivity, with a recurrence of the slow-growers constituting one-third of the resistant types, indicating that the slow-growing, resistant types are double mutants. The slow-growing segregants were also observed among the progeny of crosses involving other combinations of alleles at *act-1* and *act-2* (KH 52 × KH 56, KH 54 × KH 53, KH 57 × KH 53, KH 58 × KH 53). All the slow growers were resistant, and, in all the cases, they represented about one-fourth of the progeny, as expected if slow growth is characteristic of the double mutant.

Table 4. *Mycelium dry weights (mg) of four actidione genotypes in medium N with different concentrations of actidione**

Genotype	Actidione concentration (μg./ml.)																
	0	0.1	0.2	0.3	0.4	0.5	1	2	4	10	25	50	100	200	300	400	500
Wild-type	90	64	43	28	22	18	9	3	0	0	—	—	—	—	—	—	—
<i>act-1</i>	93	—	—	—	—	—	—	—	—	56	34	22	12	10	3	2	1
<i>act-2</i>	88	—	—	—	—	—	—	—	—	71	38	24	14	10	3	2	2
<i>act-1; act-2</i>	16	—	—	—	—	—	—	—	—	20	17	18	21	16	15	14	13

* Values are averages for three replicate flasks.

Sensitivity of homokaryons. The scoring for resistance and sensitivity was carried out on minimal slants into which actidione had been added at the concentration of 10 μg./ml. A clear-cut qualitative difference could be detected this way without paying special attention to the size of inoculum or time of scoring, since the sensitive strains did not adapt appreciably. This was true when either *act-1* or *act-2* strains were compared with the wild-type strains. Between *act-1* and *act-2*, however, there appeared to be little difference in sensitivity to the antibiotic. Quantitative measurement was accomplished by dry-weight assays in a series of actidione concentrations. In addition to wild type (Pa) and the single mutants (KH 52 and KH 53), the double mutant (KH 52; KH 53) was also tested (Table 4). The results showed that while the concentrations the single mutants can withstand are about the same, they are roughly a hundred times higher than those allowing detectable growth of wild type. The double mutant produces approximately as much mycelium in the presence of actidione as in the absence within the concentration ranges tested. The low mycelium production in liquid minimal medium by the double mutant corresponds to its slow growth on minimal agar.

Sensitivity of heterokaryons. Three types of heterokaryons were made: (1) *act-1* with ∇ , (2) *act-2* with ∇ , and (3) *act-1* with *act-2*. The alleles KH 52 and KH 53 were used. The auxotrophic alleles used in establishing all three types of heterokaryons were *pan-1* (pantothenic-1, 5531), *inos* (inositol, 37401), and *nt* (nicotinic-tryptophan, C 86). The component nuclei of some heterokaryons were also marked with either *al-2* (albino-2, 15300) or *ylo* (yellow, Y30539y).

The sensitivity of any particular heterokaryon was tested on minimal slants containing actidione. It was found that both *act-1* with ∇ and *act-2* with ∇ heterokaryons consistently expressed the resistant phenotype when they were tested on actidione. This suggested that both *act-1* and *act-2* are dominant over their corresponding wild-type alleles. The dominance was further supported by the quantitative data obtained from dry-weight assays. As shown in Table 5, the heterokaryons

of *act-1* with $\neq$ and of *act-2* with $\neq$ produced almost as much mycelium in actidione as did the *act-1* and *act-2* homokaryons.

The *act-1* with *act-2* heterokaryons were resistant in phenotype, as would be expected if the mutant genes constituting these heterokaryons are dominant. This demonstrates that at least one of the two mutant genes shows a high degree of dominance, i.e. that it is not just partially dominant over its $\neq$ allele.

Table 5. *Production of mycelium by various actidione genotypes in medium N with and without actidione*

Genotype	Phenotype†	Dry wt. of mycelium (mg.) in minimal medium*	
		Without	With
		actidione	10 µg./ml. actidione
Homokaryons			
Wild-type	S	113	0
<i>act-1</i>	R	108	85
<i>act-2</i>	R	110	86
<i>act-1; act-2</i>	R	18	18
Heterokaryons			
<i>act-1; pan-1(a) + al-2; inos(a)</i>	R	103	73
<i>act-1; nt(A) + al-2; pan-1(A)</i>	R	106	80
<i>act-2; pan-1(a) + ylo; inos(a)</i>	R	101	75
<i>act-2; pan-1(a) + al-2; inos(a)</i>	R	107	71
<i>act-1; nt(A) + act-2; ylo; inos(A)</i>	R	95	72
<i>act-1; nt(a) + act-2; pan-1(a)</i>	R	95	73

* Values are averages for two replicate flasks.

† As scored in 3 inch minimal slants containing 10 µg./ml. actidione. S = Sensitive, R = resistant.

DISCUSSION

The occurrence of a dominant mutation within a single nucleus in a multinucleate conidium produces a heterokaryotic conidium capable of giving rise to a culture with the dominant phenotype. In the present study, heterokaryotic conidia containing both resistant and sensitive nuclei can be picked up from the actidione plates because the alleles for resistance are dominant. On the other hand, if the mutant allele for resistance were recessive, the presence of such an allele in a multinucleate conidium would be phenotypically sensitive, and would, therefore, not be able to grow on the selective medium containing the antibiotic. The method used thus appears to favour the recovery of dominant, resistant mutations. The recovery of recessive mutant alleles for resistance on the selective medium would be possible under the condition that in a single conidium only the nucleus carrying the mutant allele survived u.v.-irradiation. The present result that resistant mutants are dominant does not, therefore, justify the conclusion that recessive mutants conferring resistance do not occur. In fact, recessive resistant mutants are not uncommon. For example, one of the three mutant alleles for acriflavine resistance reported in *Aspergillus nidulans* is a recessive allele (Roper & Käfer, 1957), and, by use of heterogenotes, phage resistance (Lederberg, 1949) and streptomycin resistance (Lederberg, 1951) in *Escherichia coli* have been shown to be recessives.

The mutant genes at both the *act-1* and *act-2* loci are phenotypically indistinguishable. The dominant nature of these mutants prevents testing for allelism in heterokaryons. The applicability of such dominant alleles for resistance to the automatic selection of mitotic segregants arising from diploid nuclei heterozygous for them, if a parasexual cycle does exist in *Neurospora crassa*, would depend upon whether the vegetative segregants can be distinguished from the diploid heterozygotes with respect to level of resistance. Such dominant mutant alleles would not give rise to the pseudo-wild types described by Mitchell, Pittenger & Mitchell (1952) and by Pittenger (1954), since, if the presumed heterozygous disomic strains do arise from a cross, they would be mutant in phenotype.

The presence of at least one wild-type allele at either one of the two actidione loci in a single nucleus appears to be essential for normal growth in the absence of actidione, although when both wild-type alleles are substituted by the mutant alleles, a higher degree of resistance results. This is inferred from the observation that the double mutant is slow-growing while the heterokaryon *act-1* + *act-2* is not.

The actidione-resistant mutants should prove to be useful markers in recombination studies, for they are located in well-marked chromosome arms (Perkins, 1959; Strickland, Perkins & Veatch, 1959), and the scoring for the difference in resistance and sensitivity is clear-cut. Their biochemical and physiological actions, and usefulness in other respects, largely remain to be explored.

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