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Genetic analysis of actidione resistance in *Saccharomyces cerevisiae*

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1. INTRODUCTION

Actidione, a drug produced by *Streptomyces griseus* (Whiffen, Bohonos & Emerson, 1946), inhibits the growth of fungi generally (Ford & Leach, 1948). Resistance to the drug is reported to be under the control of nuclear genes in the moulds *Aspergillus* and *Neurospora* (Warr & Roper, 1964; Hsu, 1963). Middlekauf *et al.* (1957) were the first to analyse a resistant strain of *Saccharomyces cerevisiae* and concluded that a single dominant gene controlled the character. The maximum degree of resistance conferred by this gene is not specified but their results indicate resistance to at least 2 p.p.m. of the drug.

Four strains of yeast, each with a different resistance gene, are now available and were kindly sent us by Dr D. C. Hawthorne. They are numbered 34548, 1540–16C, 1446–126B, and 138–3B, and are listed by him as carrying the genes AC_1^r, ac_2^r, AC_3^r, and AC_4^r respectively. In the report of the Yeast Genetics Conference (see *Microbial Genet. Bull.*, supplement to no. 19) the first of these genes is recorded as a recessive but our results do not confirm this.

The present series of investigations was designed to give an account of resistance genes from the point of view of their mutation rates, their individual levels of resistance, whether these levels are strain dependent, and their interaction patterns in building up resistance levels.

2. MATERIALS AND METHODS

The strains listed above, together with one (22017) of unknown genotype, were used in conjunction with sensitive strains from this laboratory (Table 1). A total of six unlinked marker genes were also available in these strains and generally showed a 2:2 segregation in dissected asci.

Resistance levels were determined both on solid and in liquid medium. In the case of solid medium, cells of the strain under test were suspended in water and dropped out on a yeast-extract, peptone agar (YEP) containing the drug in concentrations of 0·5, 1, 2, 6, 10, 20, 30, 50, 100, 200, 500, and 1000 p.p.m. Strains listed as being resistant to one of these concentrations are unable to grow, and cells do not form buds,

Table 1. *Resistance of various strains to actidione*

Strain and origin	Gene	Resistance (p.p.m.)	Resistance of heterozygote
34548 (Lindegren)	AC_1^r	1	0·5
1450–16C (Hawthorne)	ac_2^r	6	0
1446–126B (Hawthorne)	AC_3^r	1	0·5
1380–3B (Hawthorne)	AC_4^r	0·5	0·5
22017 (Lindegren)	AC_5^r	1	0·5
49–2A (derived from 1446–126B)	ac_6^r	0·5	0
1–9C (Lee)	+	0*	
22016 (Lee)	+	0*	

* Sensitive strain showing no growth on 0·5 p.p.m. after 5 days' incubation. This applies to all strains described as 'sensitive' in the text.

on the next highest concentration. Suspension drops contained approximately 10^5 cells, and plates were scored during the course of 8 days' incubation at 30°C. (Fig. 1). The same degree of tolerance was seen if cells were plated, that is uniformly spread over the agar surface rather than mass inoculated. The method of plating was used for the selection of mutant resistant cells in selection experiments.

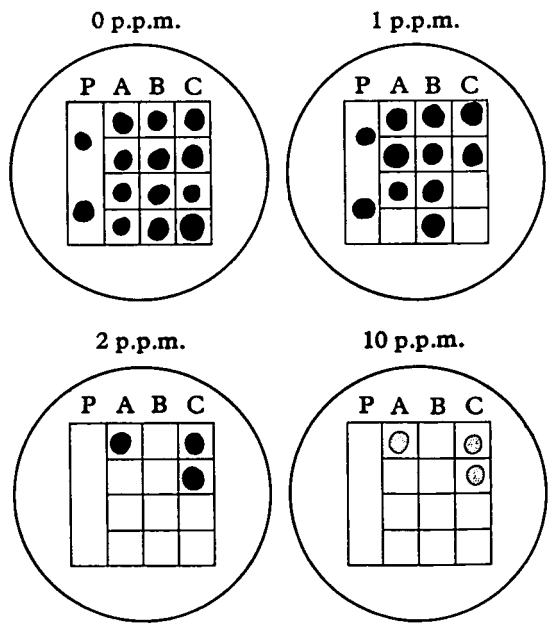

Fig. 1. Tetrad analysis by the drop-out method of the cross between strains 34584 and 22017. Drawings of petri dishes after 5 days' incubation at 30°C. Successive dishes have actidione concentrations 0, 1, 2 and 10 p.p.m., as indicated.

Vertical columns: P = parental strains.
A, B and C = tetrads.

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Liquid medium was the synthetic complete medium of Wickerham (1946) and growth rates of cultures grown aerobically on a shaker at room temperature, were measured in the Hilger colorimeter as photodensity (Fig. 2).

Stock solutions of actidione were seitz-filtered and, in the case of solid medium, appropriate amounts added to the molten agar before pouring.

Ultra-violet light was provided by the Phillips 6-W. TUV lamp using the procedure described in Wilkie (1963). In all irradiation experiments $2{\cdot}6 \times 10^4$ erg/cm.^{2} was delivered, resulting in about 10% survival.

3. RESULTS

(i) *Sensitivity and resistance levels of individual strains*

Normal, sensitive strains were found to be completely inhibited at a concentration of 0·5 p.p.m. of actidione both in liquid and on solid medium. It is likely that lower concentrations than this can stop growth of sensitive cells but these were not tested (see Middlekauf *et al.*, 1957). The effect of the drug is irreversible, and after 48 hours' exposure only 10% of sensitive cells are able to recover, while after 5 days all cells are effectively killed.

Growth curves of three strains resistant to 1, 10, and 1000 p.p.m. respectively are given in Fig. 2. In all cases the initial inoculum gave a density of 6×10^4 cells/ml. It can be seen that resistant cells grow in the presence of the drug following an initial lag and, in general, the higher the level of resistance the greater the lag at maximum tolerance. The extent of the lag period in liquid medium follows closely the delay in the appearance of colonies on agar at the same concentrations of the drug. (In these experiments the measurement of growth rate in the colorimeter is valid since optical density is proportional to cell concentration at least over the range that is of importance.)

(ii) *Individual resistance genes*

The resistance levels of the original strains were determined and are shown in Table 1. Results of crosses between strain 22017 and the known resistant strains established the presence of a fifth gene designated AC_5^r, in this strain (Table 2, cross 1). Analysis of a cross between 1446–126B and the sensitive strain 1–9C allowed the detection of a sixth gene ac_6^r in addition to AC_3^r known to be carried by 1446–126B (see also Table 2, cross 2).

Each of the stock resistant strains was crossed to a sensitive strain (1–9C or 22016 depending on mating type required) and individual progeny tested for resistance. A strict 2:2 segregation of resistance:sensitivity was seen in the tetrads of all derived diploids with resistant progeny showing the same level of resistance as the respective resistant parent. As well as showing single-gene determination of the resistance in each case, these results also indicated that the expression of the resistance genes was independent of genetic factors of the respective sensitive strains. In these crosses a total of sixty-two asci were dissected and analysed.

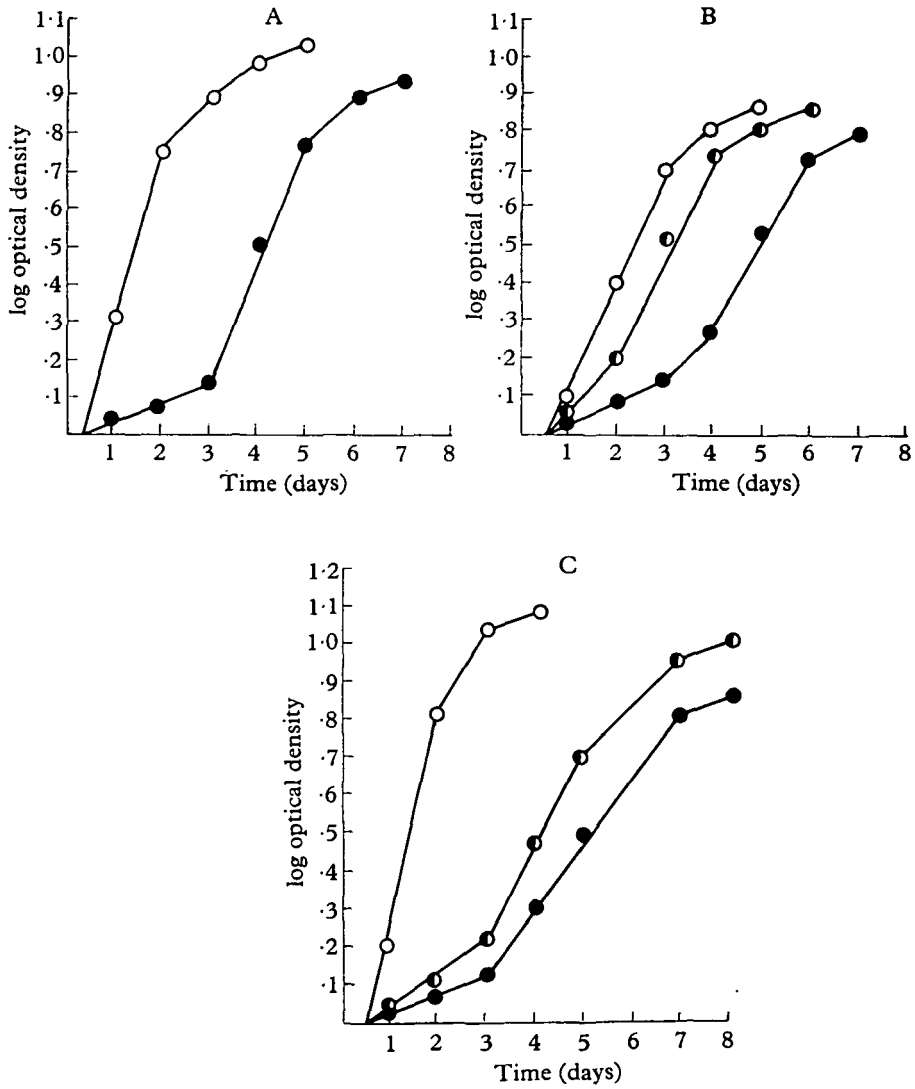

Fig. 2. Growth curves of strains resistant to actidione.

A. Strain 22017 (carries AC_6^r and resistant to 1 p.p.m.).

●, growth in 1 p.p.m. actidione–YEP.
○, growth in YEP.

B. Strain 22015 (carries Ac_1^r and AC_5^r, resistant to 10 p.p.m.).

◐, growth in 2 p.p.m. actidione–YEP.
●, growth in 10 p.p.m. actidione–YEP.
○, growth in YEP.

C. Strain 63–2 (carries AC_7^r, ac_8^r and m_7; resistant to 1000 p.p.m.).

◐, growth in 200 p.p.m. actidione–YEP.
●, growth in 1000 p.p.m. actidione.–YEP.
○, growth in YEP.

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Table 2. *Tetrad analysis of crosses between actidione-resistant strains*

Cross 1. 34548 R_1 (1 p.p.m.) × 22017 R_5 (1 p.p.m.)

Resistance pattern of tetrads and genotypes*

10	R_1R_5	10	R_1R_5	1	R_1+
1	R_1+	10	R_1R_5	1	R_1+
1	$+R_5$	0	$++$	1	$+R_5$
0	$++$	0	$++$	1	$+R_5$
No.	8		2		1

Cross 2. 1446–126B R_3r_6 (2 p.p.m.) × 38–3A R_1 (1 p.p.m.)

Resistance pattern of tetrads and genotypes*

30	$R_1R_3r_6m_1$	30	$R_1R_3r_6m_1$	10	$R_1R_3+m_1$	6	$R_1+r_6m_1$
6	$R_1+r_6m_1$	2	R_1++m_1	6	$R_1+r_6m_1$	6	$R_1+r_6m_1$
1	$+R_3++$	2	$+R_3r_6+$	1	$+R_3++$	1	$+R_3++$
0	$++++$	0	$++++$	0·5	$++r_6+$	1	$+R_3++$
No.	2		1		1		1

Cross 3. 13–2D $R_1R_3r_6m_1$ (30 p.p.m.) × 1450–6C r_2 (6 p.p.m.)

Resistance pattern of tetrads

	200	100	50
	200	30	50
	2	10	10
	1	2	2
No.	2	5	1

* R, r, abbreviations of AC^r, ac^r; m, modifying gene.

The degree of resistance of heterozygous diploids (Table 1) indicated that the genes AC^r_1, AC^r_3 and AC^r_5 are semi-dominant, that ac^r_2 and ac^r_6 are recessive, and that AC^r_4 is dominant.

(iii) *Combinations of resistance genes*

Tetrad analysis of crosses between resistant strains (Table 2) resulted in one class of recombinant of higher resistance level than parental types. These recombinants were shown to carry more than one resistance gene and in most cases positive interaction was seen. Thus, for example, the gene combinations $AC^r_1\,AC^r_5$ and $AC^r_4\,AC^r_5$ had resistance levels of 10 p.p.m. and 6 p.p.m. respectively.

In some crosses between resistant strains (e.g. Table 2, cross 2) tetrad segregations were obtained which could not be explained in terms of recombination of resistance genes alone and the presence of specific modifier genes was deduced in these cases. Such genes do not themselves confer resistance but are believed to affect the expression of resistance genes. In the particular case of the recombinant of postulated genotype $AC^r_1 m_1$, the presence of a recessive, unlinked modifier gene, m_1, was established from the analysis of a cross to the sensitive strain 1–9C in which the expected segregations of 2 p.p.m.—resistant, 1 p.p.m.-resistant, and sensitive progeny were obtained. In a further case, details of which are given in the section that follows, another modifying gene m_7, has been identified and found to increase resistance five-fold in certain resistant cells.

(iv) *Progressive selection for actidione resistance*

First-step resistant mutants were selected from sensitive cells plated on a medium containing 1 p.p.m. actidione and tested for their maximum resistance on plates with higher concentrations of the drug. The highest level of resistance seen in a first-step mutant was 20 p.p.m. out of twenty that were tested.

Second-step mutants were selected from cells of first-step mutants by plating on yet higher concentrations, and so on for third- and fourth-step mutants. Selection of this kind clearly favours multigenic systems with positive interaction between individual genes (see also Cavalli & Maccacaro, 1952).

The discontinuous, step wise increase in resistance found in four different series is set out below, with each step representing the highest level of resistance obtained from a sample of 10^7 cells in the spontaneous series and 10^6 viable cells in the ultra-violet series.

1. Spontaneous: $0 \rightarrow 1$ p.p.m. $\rightarrow 2$ p.p.m. $\rightarrow 30$ p.p.m. $\rightarrow 100$ p.p.m.
2. Ultra-violet induced: $0 \rightarrow 1$ p.p.m. $\rightarrow 200$ p.p.m. $\rightarrow 1000$ p.p.m.
3. Ultra-violet induced: $0 \rightarrow 1$ p.p.m. $\rightarrow 50$ p.p.m. $\rightarrow 100$ p.p.m. $\rightarrow 200$ p.p.m.
4. Ultra-violet induced: $0 \rightarrow 20$ p.p.m. $\rightarrow 50$ p.p.m. $\rightarrow 500$ p.p.m. $\rightarrow 1000$ p.p.m.

In the case of series 2 each successive (induced) mutant was crossed to two different sensitive strains numbered respectively 121–4A and 11.

A 2:2 segregation of 1 p.p.m.: sensitive was seen in all asci from the cross between the first-step mutant and 121—4A. This showed control by a single gene which, from the resistance level of the heterozygous diploid (0·5 p.p.m.) was semi-dominant and was tentatively designated AC_7^r. On the other hand, ascus analysis of the cross between the first-step mutant and strain 11 yielded tetrads with resistance distributions as follows: (*a*) 2 p.p.m., 2 p.p.m., 0, 0 (1 ascus); (*b*) 2 p.p.m., 1 p.p.m., 0, 0 (5 asci); (*c*) 1 p.p.m., 1 p.p.m., 0, 0 (1 ascus). From these results it was concluded that strain 11 carried an unlinked, recessive modifying gene, designated m_7, specific to AC_7^r such that the presence of both these genes in a cell conferred resistance to 2 p.p.m. This was verified by crossing the 2 p.p.m.-resistant recombinant to the sensitive strain 121–4A with the expected results on tetrad analysis.

The second-step mutant resistant to 200 p.p.m. gave a diploid resistant to 0·5 p.p.m. when crossed to 121–4A. Sporulation and tetrad analysis of this diploid gave resistance patterns as follows: (*a*) 200 p.p.m., 200 p.p.m., 0, 0 (1 ascus); (*b*) 200 p.p.m., 20 p.p.m., 1 p.p.m., 0 (5 asci). These results indicated that the second step in the series resulted from the mutation of another resistance gene (ac_8^r) which was recessive and conferred resistance to 20 p.p.m. when alone but interacted with AC_7^r to allow a tolerance of 200 p.p.m.

Analysis of the cross of the second-step mutant with strain 11 gave tetrads of the following types: (*a*) 1000 p.p.m., 1000 p.p.m., 0, 0 (2 asci); (*b*) 1000 p.p.m., 20 p.p.m., 0 (2 asci); (*c*) 1000 p.p.m., 200 p.p.m., 0, 0 (2 asci); (*d*) 200 p.p.m., 20 p.p.m., 2 p.p.m., 0 (2 asci). The recombinant resistant to 1000 p.p.m. obtained in these dissections was shown to have the genes $AC_7^r ac_8^r m_7$ from the analysis of backcrosses to each

parent and from a cross to 121–4A in all of which the expected segregations were obtained. For example, in the backcross to the 200 p.p.m.-resistant parent there was 2:2 segregation of 1000 p.p.m.:200 p.p.m. resistance in each of 5 asci tested.

The third-step mutant of series 2 (resistant to 1000 p.p.m.) was crossed to 121–4A giving a diploid resistant to 0·5 p.p.m. Recombinant progeny among the ascospores from this diploid had one or other of the resistance levels of 200 p.p.m., 20 p.p.m., 2 p.p.m., and 1 p.p.m. with tetrad patterns indicating that the third step had involved the mutation of an unlinked modifying gene thought to be m_7. This was

Table 3. *Some gene combinations and resistance conferred*

$R_1 R_5$	10	$R_1 m_1$	2
$R_3 r_6$	2	$r_2 m_2$*	20
$R_4 R_5$	6	$R_3 r_6 m_3$*	6
$r_2 r_6$	10	$R_1 r_6 m_1$	6
$R_7 r_8$	200	$R_7 r_8 m_7$	1000
$R_1 R_4 R_5$	10	$R_1 R_3 r_6 m_1$	30

* The presence of modifiers m_2 and m_3 is inferred from tetrad data.

shown to be the case by crossing the third-step mutant to the recombinant resistant to 1000 p.p.m. which was obtained following the analysis of the second-step mutant (above). This cross gave a diploid resistant to 1000 p.p.m. and all the ascospores of which were likewise resistant to 1000 p.p.m. In other words this diploid was homozygous at all three loci. Series 2 then, may be represented as follows:

Wild-type	⟶	AC_7^r	⟶	$AC_7^r ac_8^r$
(sensitive)		(resistant to 1 p.p.m.)		(resistant to 200 p.p.m.)

⟶	$AC_7^r ac_8^r m_7$	
	(resistant to 1000 p.p.m.)	

4. DISCUSSION

A multigenic system of resistance to actidione operates in yeast and resistance genes are probably numerous. Cavalli & Maccacaro (1952) describe a polygenic system of resistance to chloramphenicol in *Escherichia coli*. In their analysis of crosses between highly resistant strains they found that the progeny never showed resistance higher than that of the parent strains. They concluded that this may be due to negative interaction between resistance genes but more likely to the presence of modifier genes in the system. The presence of specific modifier genes has been clearly demonstrated in the present investigation and some of the gene combinations obtained are listed in Table 3 together with their actidione tolerance.

In her studies on streptomyces resistance in *Pneumococcus*, Bryan (1961) has shown there is positive interaction between resistance genes. In transformation experiments donor DNA from cells carrying an 'enhancement' factor *en*, which apparently confers resistance to 300 p.p.m. when alone in the cell, increased ten-fold the resistance of recipient, resistant cells carrying an allele of the *str-r* series.

These two cases of drug resistance in bacteria have been cited to bring out apparent similarities to yeast in the genetic control of resistance. There remains a great deal of literature on resistance to antibiotics in bacteria, particularly to penicillin and streptomycin, and a general comparison with the present findings will not be attempted. Indeed, until something is known of the mechanism of gene action in actidione resistance such comparisons are probably premature. In attempting to solve this problem of mechanism of resistance, actidione derivatives (one of them radioactive) have been prepared in this laboratory and their effects in both sensitive and resistant cells studied (Lee & Wilkie, 1964). It is hoped that the analysis of fraction of cells fed with radioactive analogues will provide further information on the mode of action of resistance genes.

SUMMARY

1. Individual genes of a multigenic system of resistance to actidione in yeast were analysed in a series of crosses involving resistant strains and sensitive strains.

2. Resistance conferred varied in the range 0·5 p.p.m. to 20 p.p.m.

3. Positive interaction was seen between resistance genes in recombinant strains.

4. Recessive modifier genes were found which do not themselves confer resistance but which modify the activity of specific resistance genes in a positive way, that is towards a greater degree of resistance. Both a two-fold and a five-fold increase in resistance is seen depending on the gene combination.

5. In selection experiments, strains resistant to relatively high concentrations of actidione (1000 p.p.m.) were obtained but only by selection on media containing successively higher concentrations of the drug, that is, by the step wise build up of multiply mutant forms. Mutations of interacting resistance genes and specific modifier genes were obtained by selection methods including pretreatment of cells with ultra-violet light.

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