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A genetic analysis of resistance to nystatin in *Saccharomyces cerevisiae*

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1. INTRODUCTION

Nystatin is a polyene antifungal agent produced by *Streptomyces noursei* (Hazen & Brown, 1951). Although polyenes have been used for some time to control fungal infections in clinical practice and in the laboratory there have been few reports of fungi developing resistance to them. A number of workers have attempted to obtain resistant strains but in general have not succeeded in raising the level of tolerance more than two or three fold (Stout & Pagano, 1956; Littman *et al.*, 1958; Manning & Robertson, 1959; Stanley & English, 1965). Recently Hebeka & Solotorovsky (1965) isolated strains of *Candida albicans* with markedly increased resistance to the polyenes candidin (150 fold) and amphotericin B (60 fold) but were unable to increase resistance to nystatin more than three fold.

We have isolated nystatin-resistant strains of *Saccharomyces cerevisiae* and used these mutants to investigate the genetic basis of resistance to nystatin. This paper presents data supporting the identification of three genes, *nys*-1, *nys*-2 and *nys*-3, which confer resistance to nystatin, and two modifying genes, M^{nys}-1 and M^{nys}-2, which enhance the effects of the resistance genes but which do not confer resistance themselves.

2. MATERIALS AND METHODS

The complete and sporulation media were those listed by Cox & Bevan (1962). The minimal medium was Difco Bacto Yeast Nitrogen Base w/o Amino Acids, with 4% glucose as the carbon source. These three media were adjusted to pH 5·8 and solidified with 1·5% Oxoid No. 3 Agar.

Nystatin (Mycostatin-Squibb) was purchased from E. R. Squibb & Sons Ltd. as a sterile powder in vials containing 500,000 units at 3000 units/mg. The contents of a vial were suspended in 100 ml. of sterile water, dispensed in 10-ml. aliquots into 1 oz. containers and stored at −25°C. for not more than 6 weeks. Before use the suspensions were allowed to thaw at room temperature, appropriate volumes were then added to medium at 40°C. and plates poured immediately.

Two wild-type strains of mating types *a* and *α* were used throughout the work. All of the mutants resistant to nystatin were isolated from wild-type *a*. Stock cultures of mutants and wild-types were maintained on complete medium at 3–6°C. and subcultured at intervals.

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Incubation was at 28°C. on complete medium with or without added nystatin except in those experiments to investigate the effect of temperature and minimal medium on resistance.

Haploid strains of opposite mating types were crossed by mixing suspensions on complete medium, isolating mating figures after 2–3 hours and removing presumptive diploid cells to prepared positions on the same plate after a further 2–3 hours of incubation. Diploid cultures were induced to sporulate by transfer to sporulation medium after overnight growth on complete medium. Cultures on sporulation medium were incubated at 28°C. and examined for the presence of asci after 3 days. Asci were treated prior to dissection with an extract of the fruiting bodies of *Agaricus campestris* var. *hortensis* which digests the ascus walls (Bevan & Costello, 1964).

Matings and ascus dissections were carried out by micromanipulation on open plates (Kemp & Bevan, 1959). Mating type was determined by an adaptation of the technique used by Bevan & Woods (1962) for adenine-requiring mutants. Segregants were replica-plated on to plates spread with lawns of wild-types *a* and *α*. The plates were examined for the presence of mating figures after 3 hours' incubation.

Levels of resistance were determined by replica plating on to medium containing various concentrations of nystatin. All tests for resistance were duplicated and when possible the arrangements of inocula on the duplicates were randomized. Control experiments showed that nystatin did not deteriorate appreciably until the third day of incubation at 28°C.; accordingly all results were recorded after 3, 4 and 5 days. Tests for resistance were made on 10, 15, 20, 25, 30, 35, 40, 50, 60 units and higher concentrations of nystatin when appropriate. Throughout this paper we shall refer to resistance in terms of units/ml. of nystatin; by this we mean the highest concentration on which growth is visible after 4 days' incubation. In some instances two phenotypes, one more vigorous than the other, could be distinguished on 10 units/ml. but both failed to grow on 15 units/ml. To accommodate this we have denoted the more vigorous phenotype '10^{+}'. For convenience we have considered the resistance of wild-types *a* and *α* and other equally sensitive diploids and segregants as zero although they are resistant to 5 units/ml.

Only complete tetrads showing normal segregation for mating type were used in the compilation of results.

3. RESULTS

(i) *Isolation and characterization of mutants*

Apart from the use of yeast as our experimental material our procedure differed from that of previous workers in two respects: (*a*) we incubated cultures at 28°C. rather than at 37°C., (*b*) we did not dissolve the nystatin in a solvent but added it to the medium as a suspension. Accordingly we set up experiments to test our system. Gradient plates (Szybalski, 1952) showed that neither wild-type strain grew beyond approximately 5 units/ml. but that wild-type *a* was slightly more

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resistant than wild-type α. Plating experiments showed that no cells of wild-type *a* survived on 6 units/ml. and that the LD-50 was 5 units/ml. Cells of wild-type α grew up to 4 units/ml. and the LD-50 was 3·5 units/ml. These results were in sufficient accord with those published for yeast that we felt justified in omitting the use of solvents and keeping to our normal cultural conditions.

Resistant mutants were selected by spreading wild-type *a* on a range of nystatin concentrations from 10 to 20 units/ml. at intervals of 2 units/ml. using 10^6 cells

Table 1. *Resistance levels of first-step mutants*

Group	Mutants	Resistance (units/ml.)
I	10.1, 12.4	60
II	10.2, 10.4, 10.5, 10.6, 10.8, 10.9, 12.6, 12.7, 16.1	15
III	10.7, 10.10, 10.11, 12.1, 12.2, 12.3, 12.5, 12.8, 12.9, 12.10, 14.2, 20.1	10^+

Table 2. *Preliminary analysis of first-step mutants*

Group	Mutants	Resistance	Resistance of segregants	Types of tetrad	Numbers of tetrads
I	10.1, 12.4	60	60, 30, 20, 0	60, 60, 0, 0	7
				60, 20, 0, 0	6
				30, 30, 0, 0	9
				20, 20, 0, 0	16
II	10.2, 10.4, 10.5,	15	15, 10, 0	15, 15, 0, 0	22
	10.6, 10.8, 10.9,			15, 10, 0, 0	42
	12.6, 12.7, 16.1			10, 10, 0, 0	23
III	10.7, 10.10, 10.11,	10^+	10^+, 10, 0	10^+, 10^+, 0, 0	29
	12.1, 12.2, 12.3,			10^+, 10, 0, 0	56
	12.5, 12.8, 12.9,			10, 10, 0, 0	31
	12.10, 14.2, 20.1				

per plate. The mutants isolated from these plates, their nomenclature and their levels of resistance, are listed in Table 1. The mutants are numbered according to the nystatin concentration from which they were isolated, e.g. 10.1, 10.2, etc. from 10 units/ml.; 12.1, 12.2, etc. from 12 units/ml. and so on.

The mutants were next crossed to wild-type α to obtain resistant segregants in both mating types for further analysis. Diploid heterozygotes from these crosses were tested for resistance; none were more resistant than wild-type *a* indicating that the mutants are recessive. The segregants were tested on nystatin concentrations from 10 to 80 units/ml.

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Although resistance versus sensitivity segregated 2:2 in all of the crosses the levels of resistance were variable in any particular cross. For example, mutant 10.1, resistant to 60 units/ml., gave segregants resistant to 20, 30 and 60 units/ml. A total of three patterns of resistance was found. The mutants exhibiting these patterns and the types of tetrad observed are shown in Table 2, mutants showing the same pattern of resistance have been grouped.

(ii) *Functional analysis of the mutants*

Segregants in both mating types showing the resistance level characteristic of the original mutants were selected, crossed in all of the possible pairwise combinations and the resulting diploids tested on nystatin medium. Since the mutants

Table 3. *Allocation of resistant mutants to genes* nys-1, nys-2 *and* nys-3

Gene	Mutants
nys-1	10.1, 12.4
nys-2	10.2, 10.4
nys-3	10.5, 10.6, 10.7, 10.8, 10.9, 10.10, 10.11, 12.1, 12.2, 12.5, 12.6, 12.7, 12.8, 12.9, 12.10, 14.2, 16.1, 20.1

Table 4. *The results of crosses between mutants of genes* nys-1, nys-2 *and* nys-3*

Cross	Resistance of diploid	Asci tested	Resistance and frequency of segregants
nys-1 × *nys*-1	60	27	60 (108)
nys-2 × *nys*-2	15	19	15 (76)
nys-3 × *nys*-3	15	18	15 (72)
nys-1 × *nys*-2	0	14	120 (9), 60 (19), 15 (19), 0 (9)
nys-1 × *nys*-3	0	16	120 (16), 60 (16), 15 (16), 0 (16)
nys-2 × *nys*-3	0	23	25 (21), 15 (50), 0 (21)

* All strains carry M^{nys}-1 and M^{nys}-2 (see later).

were known to be recessive, allelic mutants were expected to give a resistant diploid and non-allelic mutants a sensitive diploid. As a further test some diploids were sporulated and dissected to check for the occurrence of sensitive segregants. These should occur with predictable frequencies if the mutants crossed are non-allelic and unlinked. In Table 3 we show the allocation of the mutants to three genes, *nys*-1, *nys*-2 and *nys*-3 on the basis of the tests for allelism. Both of the mutants in Group I of Table 2 are allocated to *nys*-1; the two allocated to *nys*-2 are from Group II whereas mutants from Groups II and III classify as *nys*-3.

Table 4 lists the results of meiotic analysis of the possible inter and intragenic crosses; *nys*-1 and *nys*-2 appear to be linked. The numbers of parental ditype, non-parental ditype and tetratype asci were 5, 0 and 9 respectively. The deviation

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of the PD:NPD ratio from 1:1 is significant at the 5% level (Perkins, 1953). Random strand analysis gives a recombination frequency of 32%.

There is no evidence of linkage between *nys*-1 and *nys*-3 or *nys*-2 and *nys*-3.

The recovery of segregants which are more resistant than either parental strain from the intergenic crosses indicates that the resistance genes are additive in their effects.

(iii) *Interrelationships of the basic genes and modifiers*

The segregants from crosses of the two *nys*-1 mutants to wild-type α show three levels of resistance, the highest corresponding to that of the original mutants. This result can be explained by proposing that all resistant segregants carry *nys*-1 but that the level of resistance is determined by two modifying genes M^{nys}-1 and M^{nys}-2. Of these two M^{nys}-1 is specific for *nys*-1 and raises the resistance from 20 to 30 units/ml. whilst M^{nys}-2 is specific for the combination *nys*-1, M^{nys}-1

Table 5. *Relationships between genotype and level of resistance for* nys-1

Genotype	Resistance	Expected frequency	Observed frequency
nys-1 *M*-1 *M*-2	60	19	20
nys-1 *M*-1 +	30	19	18
nys-1 + *M*-2; *nys*-1 + +	20	38	38
+ *M*-1 *M*-2; + *M*-1 +; + + *M*-2; + + +	0	76	76

raising this to 60 units/ml., but does not affect *nys*-1 in the absence of M^{nys}-1. The original mutants, induced in wild-type *a*, have the genotype *nys*-1, M^{nys}-1, M^{nys}-2, and were crossed to wild-type α which has the genotype *nys*-1^{+}, M^{nys}-1^{+}, M^{nys}-2^{+}. From this cross we would expect the genotypes and resistance levels listed in Table 5. For convenience in the tables and some parts of the text we have used *M*-1 and *M*-2 to denote M^{nys}-1 and M^{nys}-2. It can be seen that the agreement between the observed results and those expected on the basis of free recombination between the basic and modifying genes is excellent.

The absence of two types of tetrad (60, 30, 0, 0 and 30, 20, 0, 0) in the crosses of mutants allocated to *nys*-1 (Table 2) indicates a non-random segregation of *nys*-1, M^{nys}-1 and M^{nys}-2. The data in Table 5 show that this cannot be due to linkage.

The high proportion of asci (16/38) with the patterns 60, 60, 0, 0 and 30, 30, 0, 0 and which are consequently parental or non-parental ditype for all three genes could be accounted for if all three were centromere linked. These asci would be those showing first-division segregation. Analysis of the segregation of *nys*-1 and M^{nys}-1 shows that 32/38 asci are PD or NPD and show first-division segregation

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for these two genes. This suggests that both are strongly linked to centromeres. Mating type is also centromere linked (Hawthorne & Mortimer, 1960). Mating type and *nys*-1 give 24/38 PD or NPD asci, as also do mating type and M^{nys}-1. The percentages of apparent second-division segregation, 36·8%, for both gene pairs, correlates well with the published figure for mating type, 39·9%, suggesting again that *nys*-1 and M^{nys}-1 are close to their respective centromeres. It is not possible to make a similar analysis for M^{nys}-2 but the data suggest that it is also centromere linked.

Segregants from the mutants in Groups II and III of Tables 1 and 2 show only two levels of resistance, the highest corresponding to that of the original mutant. This could be accounted for by a single modifying gene. As we shall show later this gene is M^{nys}-2, M^{nys}-1 having no effect on mutants in these groups whether

Table 6. *Relationships between genotype and level of resistance for* nys-2 *and* nys-3

Genotype	Resistance	Expected frequency	Observed frequency
nys-2 *M*	$10^+/15$	35	33
nys-2 +	10	35	37
+ *M* } + + }	0	70	70
nys-3 *M*	$10^+/15$	203	204
nys-3 +	10	203	202
+ *M* } + + }	0	406	406

they have been allotted to *nys*-2 or *nys*-3. The scheme for these mutants is set out in Table 6. Again the expected and observed results are in excellent agreement and we can consider the hypothesis to be highly plausible.

(iv) *Proof of the basic gene and modifier hypothesis*

(*a*) **Genes** *nys-1*

Four types of tetrad were found out of the six theoretically possible. Considering those with the resistance patterns (*a*) 60, 60, 0, 0, (*b*) 30, 30, 0, 0, (*c*) 20, 20, 0, 0, they can have the following genotypes:

(*a*) (1) *nys*-1 *M*-1 *M*-2
nys-1 *M*-1 *M*-2
\+ + +
\+ + +

(*b*) (1) *nys*-1 *M*-1 +
nys-1 *M*-1 +
\+ + *M*-2
\+ + *M*-2

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(*c*)	(1) *nys*-1 + + *nys*-1 + + + *M*-1 *M*-2 + *M*-1 *M*-2	(2) *nys*-1 + *M*-2 *nys*-1 + *M*-2 + *M*-1 + + *M*-1 +	(3) *nys*-1 + + *nys*-1 + *M*-2 + *M*-1 + + *M*-1 *M*-2

We used the proposed genotypes of segregants from tetrad types (*a*) and (*b*) firstly to test the consistency of the hypothesis for these two tetrad types and secondly to analyse the genotypes of tetrads of type (*c*). The results are set out in Tables 7 and 8 respectively. Crosses 1–4 were used to establish the genotype of several segregants from type (*a*) tetrads. In crosses 5–8 these are used to illustrate the genotypes of a type (*b*) tetrad. In crosses 9–16 (Table 8) we show the analysis of two type (*c*) tetrads, establishing that one (10.1—8a, b, c, d) is of type (*c* 3) and the other (12.4—2a, b, c, d) is of type (*c* 1).

The differential specificity of M^{nys}-1 and M^{nys}-2 is demonstrated by the results of crosses 5 and 6 in Table 7 and cross 12 in Table 8.

Table 7. *The results of sample crosses set up to test the basic gene and modifier hypothesis for* nys-1

Cross No.	Strains	Resistance	Tetrad derivation	Proposed genotype	Segregants	
					Expected	Observed
1	10.1–9a	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2	60, 30, 20, 0	60, 30, 20, 0
	×					
	wild-type α	0	—	+ + +		
2	10.1–9c	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2	60, 30	60, 30
	×					
	12.4–6d	30	30, 30, 0, 0	*nys*-1 *M*-2 +		
3	10.1–10c	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2	60, 30	60, 30
	×					
	12.4–6b	30	30, 30, 0, 0	*nys*-1 *M*-1 +		
4	12.4–11b	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2	60, 30, 20, 0	60, 30, 20, 0
	×					
	12.4–11c	0	60, 60, 0, 0	+ + +		
5	10.1–6a	0	30, 30, 0, 0	+ + *M*-2	60, 20, 0	60, 20, 0
	×					
	10.1–10c	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2		
6	10·1–6c	0	30, 30, 0, 0	+ + *M*-2	60, 20, 0	60, 20, 0
	×					
	10.1–9a	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2		
7	10.1–6b	30	30, 30, 0, 0	*nys*-1 *M*-1 +	60, 30	60, 30
	×					
	10·1–10c	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-2		
8	10.1–6d	30	30, 30, 0, 0	*nys*-1 *M*-1 +	60, 30	60, 30
	×					
	10.1–9a	60	60, 60, 0, 0	*nys*-1 *M*-1 *M*-1		

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Table 8. *Analysis of two* 20, 20, 0, 0 *asci from the cross* nys-1 × *wild-type* α*

Cross No.	Strains	Resistance	Genotype of tester strain	Segregants observed	Inferred genotype of strain tested
9	10.1–8a	0			+ *M*-1 *M*-2
	×			60, 30, 0	
	10.1—6b	30	*nys*-1 *M*-1 +		
10	10.1–8b	0			+ *M*-1 +
	×			30, 0	
	10.1–6b	30	*nys*-1 *M*-1 +		
11	10.1–8c	20			*nys*-1 + +
	×			60, 30, 20	
	10.1–10c	60	*nys*-1 *M*-1 *M*-2		
12	10.1–8d	20			*nys*-1 + *M*-2
	×			60, 20	
	10.1–10c	60	*nys*-1 *M*-1 *M*-2		
13	12.4–2b	0			+ *M*-1 *M*-2
	×			60, 0	
	10.1–9c	60	*nys*-1 *M*-1 *M*-2		
14	12.4–2c	0			+ *M*-1 *M*-2
	×			60, 0	
	10.1–10c	60	*nys*-1 *M*-1 *M*-2		
15	12.4–2a	20			*nys*-1 + +
	×			20, 0	
	wild-type α	0	+ + +		
16	12.4–2d	20			*nys*-1 + +
	×			60, 30, 20, 0	
	wild-type *a*	0	+ *M*-1 *M*-2		

* The segregants from the 20, 20, 0, 0 asci are underlined.

(*b*) *Genes nys-2 and nys-3*

The modifier affecting the mutants of these two genes could be M^{nys}-1, M^{nys}-2 or a third gene M^{nys}-3. We have shown that wild-type *a* was + M^{nys}-1 M^{nys}-2. Considering M^{nys}-1 and M^{nys}-2 the three types of tetrad observed can have the following genotypes:

(1) Modifier M^{nys}-1 active

(*a*) Tetrad type 10, 10, 0, 0.

(i)			(ii)			(iii)		
nys	+	+	*nys*	+	*M*-2	*nys*	+	+
nys	+	+	*nys*	+	*M*-2	*nys*	+	*M*-2
+	*M*-1	*M*-2	+	*M*-1	+	+	*M*-1	*M*-2
+	*M*-1	*M*-2	+	*M*-1	+	+	*M*-1	+

(*b*) Tetrad type 15, 15, 0, 0.

(i)			(ii)			(iii)		
nys	*M*-1	*M*-2	*nys*	*M*-1	+	*nys*	*M*-1	*M*-2
nys	*M*-1	*M*-2	*nys*	*M*-1	+	*nys*	*M*-1	+
+	+	+	+	+	*M*-2	+	+	*M*-2
+	+	+	+	+	*M*-2	+	+	+

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(*c*) Tetrad type 15, 10, 0, 0.

(i)	(ii)	(iii)	(iv)
nys *M*-1 *M*-2	*nys* *M*-1 +	*nys* *M*-1 +	*nys* *M*-1 *M*-2
nys + +	*nys* + *M*-2	*nys* + +	*nys* + *M*-2
+ *M*-1 *M*-2	+ *M*-1 +	+ *M*-1 *M*-2	+ *M*-1 +
+ + +	+ + *M*-2	+ + *M*-2	+ + +

(2) Modifier M^{nys}-2 active

(*a*) Tetrad type 10, 10, 0, 0.

(i)	(ii)	(iii)
nys + +	*nys* *M*-1 +	*nys* *M*-1 +
nys + +	*nys* *M*-1 +	*nys* + +
+ *M*-1 *M*-2	+ + *M*-2	+ *M*-1 *M*-2
+ *M*-1 *M*-2	+ + *M*-2	+ + *M*-2

(*b*) Tetrad type 15, 15, 0, 0.

(i)	(ii)	(iii)
nys *M*-1 *M*-2	*nys* + *M*-2	*nys* *M*-1 *M*-2
nys *M*-1 *M*-2	*nys* + *M*-2	*nys* + *M*-2
+ + +	+ *M*-1 +	+ *M*-1 +
+ + +	+ *M*-1 +	+ + +

(*c*) Tetrad type 15, 10, 0, 0.

(i)	(ii)	(iii)	(iv)
nys *M*-1 *M*-2	*nys* + *M*-2	*nys* + *M*-2	*nys* *M*-1 *M*-2
nys + +	*nys* *M*-1 +	*nys* + +	*nys* *M*-1 +
+ *M*-1 *M*-2	+ *M*-1 +	+ *M*-1 *M*-2	+ + *M*-2
+ + +	+ + *M*-2	+ *M*-1 +	+ + +

We used strains of known genotype from the crosses involving *nys*-1 to check the segregation of M^{nys}-1 and M^{nys}-2.

In Table 9 we show the results of tests on one ascus of each type. The inferred genotypes of the segregants suggest that M^{nys}-2 is the modifier affecting *nys*-2. The analysis does not rule out the possibility of a third modifier M^{nys}-3, but we feel that in the absence of evidence to the contrary we are justified in assigning this role to M^{nys}-2. The same testing procedure demonstrated that M^{nys}-2 is also specific for *nys*-3. In all we have carried out 106 crosses and all have given results in accordance with the conclusion that M^{nys}-2 is specific for *nys*-2 and *nys*-3 and that M^{nys}-1 has no effect on either of these two resistance genes.

(v) *Dominance of the modifying genes* M^{nys}-1 *and* M^{nys}-2

In the course of the experiments to test the basic gene and modifier hypothesis several diploids homozygous for *nys*-1 but heterozygous for M^{nys}-1 and or M^{nys}-2 were synthesized. These and other diploids were routinely tested for resistance. The results suggested that the modifying genes were dominant and further diploids were synthesized to check this observation. The data collected are presented in Table 10. It can be seen that both M^{nys}-1 and M^{nys}-2 are dominant but that this does not affect the recessiveness of *nys*-1, *nys*-2 and *nys*-3.

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Table 9. *Analysis of three asci from a cross of an* nys-2 *mutant* (10.2) *to wild-type α**

Ascus	Cross No.	Strains	Resistance	Genotypes of tester strain	Segregants observed	Genotype of strain tested
10.2–4	1	10.2–4a	0			+ *M*-1 +
(15, 15, 0, 0)		×			60, 30, 0	
		10.1–9a	60	*nys*-1 *M*-1 *M*-2		
	2	10.2–4c	0			+ *M*-1 +
		×			60, 30, 0	
		10.1–10c	60	*nys*-1 *M*-1 *M*-2		
	3	10.2–4b	15			*nys*-2 + *M*-2
		×			15, 0	
		12.4–6a	0	+ + *M*-2		
	4	10.2–4d	15			*nys*-2 + *M*-2
		×			15,0	
		12.4–6c	0	+ + *M*-2		
10.2–7	5	10.2–7a	0			+ *M*-1 *M*-2
(15, 10, 0, 0)		×			60, 0	
		10.1–9c	60	*nys*-1 *M*-1 *M*-2		
	6	10.2–7d	0			+ *M*-1 +
		×			60, 30, 0	
		10.1–9c	60	*nys*-1 *M*-1 *M*-2		
	7	10.2–7b	15			*nys*-2 + *M*-2
		×			15, 0	
		12.4–6a	0	+ + *M*-2		
	8	10.2–7d	10			*nys*-2 + +
		×				
		12.4–6a	0	+ + *M*-2		
10.2–3	9	10.2–3a	0			+ *M*-1 *M*-2
(10, 10, 0, 0)		×			60, 0	
		10.1–10c	60	*nys*-1 *M*-1 *M*-2		
	10	10.2–3c	0			+ *M*-1 *M*-2
		×			60, 0	
		10.1–10c	60	*nys*-1 *M*-1 *M*-2		
	11	10.2–3b	10			*nys*-2 + +
		×			15, 10, 0	
		10.1–6a	0	+ + +		
	12	10.2–3d	10			*nys*-2 + +
		×			15, 10, 0	
		10.1–6a	0	+ + *M*-2		

* The strains tested are underlined.

(vi) *Effects of environment on resistance*

All of the work we have discussed so far was carried out with complete medium at 28°C. Previous attempts by other workers to obtain nystatin resistance have mainly used similar nutrient media but have involved incubation at 37°C. We

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Table 10. *Resistance of diploids synthesized to investigate the dominance of the modifiers* M^{nys}-1 *and* M^{nys}-2

Cross No.	Genotypes	Resistance of haploid components	Resistance of diploids
1	*nys*-1 *M*-1 *M*-2	60	60
	×		
	nys-1 + +	20	
2	*nys*-1 *M*-1 +	30	60
	×		
	nys-1 + *M*-2	20	
3	*nys*-1 *M*-1 +	30	30
	×		
	nys-1 + +	20	
4	*nys*-2 *M*-1 +	10	15
	×		
	nys-2 + *M*-2	15	
5	*nys*-2 + *M*-2	15	15
	×		
	nys-2 + +	10	
6	*nys*-3 *M*-1 *M*-2	15	15
	×		
	nys-3 *M*-1 +	10	
7	*nys*-3 + *M*-2	15	15
	×		
	nys-3 + +	10	

Table 11. *Effects of temperature and medium on resistance*

Gene	Strain	Genotype	Resistance: Complete medium 28°C.	Complete medium 37°C.	Minimal medium 28°C.	Minimal medium 37°C.
nys-1	10.1	*nys*-1 *M*-1 *M*-2	60	120	15	10
	12.4	*nys*-1 *M*-1 *M*-2	60	120	15	10
	12.4–6d	*nys*-1 *M*-1 +	30	50	10+	10
	10.1–8d	*nys*-1 + *M*-2	20	60	10+	10
	10.1–8c	*nys*-1 + +	20	40	10	5
nys-2	10.2	*nys*-2 *M*-1 *M*-2	15	30	5+	5
	10.2–7b	*nys*-2 *M*-1 +	10	20	5+	5
	10.2–4b	*nys*-2 + *M*-2	15	30	5+	5
	10.2–7c	*nys*-2 + +	10	20	5+	5
nys-3	10.6	*nys*-3 *M*-1 *M*-2	15	30	5+	5
	10.8	*nys*-3 *M*-1 *M*-2	15	25	5+	5
	12.1	*nys*-3 *M*-1 *M*-2	10+	30	5+	5
	12·6	*nys*-3 *M*-1 *M*-2	15	30	5+	5
	20.1	*nys*-3 *M*-1 *M*-2	10+	30	5+	5
	10.7–3a	*nys*-3 *M*-1 +	10	20	5+	5
	10·5–2c	*nys*-3 + *M*-2	15	30	5+	5
	10.5–10d	*nys*-3 + +	10	20	5	5
	Wild-type *a*	+ *M*-1 *M*-2	5+	10+	0	0
	Wild-type *α*	+ + +	5	10	0	0

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thought that the different incubation temperature might have some bearing on our success in obtaining resistance and so we tested our mutants and segregants at 28°C. and 37°C. on both complete and minimal media. The results of this comparative experiment are shown in Table 11.

All of the strains tested, including the two wild-types, are more resistant at 37°C. on complete medium. All of them are less resistant on minimal medium than on complete medium and less resistant on minimal medium at 37°C.

The most interesting aspect of these environmental effects concerns the specificity of M^{nys}-1 and M^{nys}-2. At 37°C. on complete medium M^{nys}-2 affects *nys*-1, in fact it is more effective than M^{nys}-1. On minimal medium M^{nys}-2 affects *nys*-1 at both temperatures and is equivalent to M^{nys}-1 in this respect.

On the other hand M^{nys}-1 has no discernible effect of *nys*-2 or *nys*-3 under any of the conditions tested.

(vii) *Isolation of second-step mutants*

By plating first-step mutants on higher concentrations of nystatin we have been able to isolate strains with increased resistance to nystatin. The results of selection experiments with five first-step mutants are shown in Table 12.

Table 12. *Characterization of some second-step mutants*

First-step mutant	Resistance	Second-step mutants	
		Resistance	Number isolated
10.1 (*nys*-1)	60	120	10
		140	12
10.2 (*nys*-2)	15	30	4
		40	5
10.4 (*nys*-2)	15	20	4
		30	3
10.5 (*nys*-3)	15	30	2
		60	2
		300	4
		800	8
10.7 (*nys*-3)	10+	30	6
		80	3
		200	3

It is thus possible to obtain mutants resistant to high concentrations of nystatin, although its limited solubility makes accurate characterization difficult. The differences in potential for the development of high resistance between *nys*-1, *nys*-2 and *nys*-3 is surprising. The resistance of second-step mutants from *nys*-1 and *nys*-2 is only double that of the first steps whereas for *nys*-3 it can be increased as much as 80 fold. As with the effect of the modifiers this potential seems to be gene specific rather than allele specific.

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4. DISCUSSION

Multigenic systems controlling antibiotic resistance appear to be common in micro-organisms and this has led to comparisons with polygenic characters in higher organisms (Cavalli & Maccacaro, 1952; Bryan, 1961). Yeast is amenable to precise genetic analysis and also has a stable diploid phase. It would be possible to use these attributes to construct and dissect a multigenic system more easily than in a bacterium and also to apply quantitative analysis to a microbial system. The genetic complexity of nystatin resistance in yeast should be well suited to such an approach.

However, we agree with Wilkie & Lee (1965) that to draw comparisons between the genetic control of drug resistance in different organisms is a sterile exercise in the absence of biochemical information on the mechanisms of resistance. Where this has been investigated, as for example penicillin resistance in *Bacillus licheniformis* (Dubnau & Pollock, 1965) and *Staphylococcus aureus* (Richmond, 1965), the genetic system is concerned with the induction and synthesis of the enzyme penicillinase and apart from the intricacies of the Jacob-Monod model is relatively simple.

Nystatin resistance may prove to be particularly interesting for the correlation of genetic and biochemical studies. A great deal of work has been done on the mechanism of action of nystatin, much of it with yeast as the experimental material. Lampen (1966) has formulated a scheme for the action of polyenes. He proposes that they complex with the cell membrane, altering its permeability, and that this leads to loss of the ability to concentrate small molecules and the leakage of K^+, sugars and other essential metabolites. Sterols in the cell membrane, probably ergosterol, have been identified as binding sites for nystatin (Lampen *et al.* 1962) and all polyene-sensitive organisms contain sterols as a cell membrane component.

The widespread consequences of the action of nystatin (Lampen, 1966) suggest that resistance could result from a number of metabolic alterations. It has been reported that stationary phase cells are more resistant than log phase cells (Marini *et al.*, 1961) and this has been related to the heavier cell wall formed from bud-scar tissue (Lampen *et al.*, 1962). Mutants simulating this ageing effect could thus give low levels of resistance. Changes in the composition of the cell membrane, particularly alterations in the ratio of phospholipid to sterol, could have similar effects.

In this context van Zutphen *et al.* (1966) have shown that synthetic lipid bilayer membranes are disrupted by polyenes but that the speed of disruption is dependent on the molar ratio of lecithin to cholesterol. Equimolar membranes were more susceptible than ones with a 10:1 ratio of phospholipid to sterol. On the other hand Lampen *et al.* (1960) tested yeast cultures grown to contain high and low levels of ergosterol for nystatin uptake and sensitivity and found no significant difference between them. However, they point out that the ergosterol content of the cells may not have reflected the content of the cell membrane.

One might consider such wall or membrane changes as possible primary causes of resistance. The modifier genes could then act by buffering the cell against the

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consequences of increased permeability, possibly by increasing the efficiency of concentration mechanisms or altering the susceptibility of key enzymes to deficiencies of cofactors, etc. Certainly it is not surprising that nystatin resistance in yeast is multigenic.

SUMMARY

1. A number of stable nystatin-resistant mutants of the yeast *Saccharomyces cerevisiae* have been isolated from platings of a sensitive wild-type strain on low concentrations of the antibiotic.

2. These mutants were found to be resistant to 10, 15 or 60 units of drug/ml.

3. Analysis of meiotic segregants from crosses of these mutants to wild-type indicate that resistance is determined by two types of genes; resistance genes and modifiers.

4. Functional analysis of the mutants demonstrated the existence of three recessive resistance genes, *nys*-1, *nys*-2 and *nys*-3 and that *nys*-1 and *nys*-2 were linked.

5. Genetic analysis showed that *nys*-1 was affected by two modifiers, M^{nys}-1 and M^{nys}-2, but that only M^{nys}-2 affected *nys*-2 and *nys*-3.

6. The modifiers M^{nys}-1 and M^{nys}-2 are dominant.

7. An investigation of the effects of temperature and medium on resistance demonstrated marked interactions between genotype and environment for both the resistance genes and the modifiers.

8. Second-step mutants have been isolated by plating first-step mutants on higher concentrations of the drug. Some of these are resistant to 800 units/ml.

9. Some possible mechanisms of nystatin resistance are discussed.

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