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Genetic analysis of nystatin-resistant mutants of *Saccharomyces cerevisiae*

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SUMMARY

Primary genes, designated as *NYSA*, *NYSD*, *NYSE*, *NYSF* (dominant) and *nysB*, *nysC* (recessive) are responsible for resistance to nystatin ranging from 30 to 80 u/ml. A dominant modifier gene increases the resistance conferred by *NYSF* from 80 to 140 u/ml and a recessive modifier gene enhances resistance due to *NYSA* by 20 u/ml (from 40 to 60 u/ml). One nystatin-resistant mutant is apparently cytoplasmic and this 'mutation' suppresses specifically the action of modifying factors which increase the level of resistance conferred by the *NYSA* gene. Interaction resulting in additive levels of resistance is shown by gene *NYSD* in combination with each of the genes *nysB*, *nysC* and *NYSE*. A model of step-wise increases in resistance due to polygenic primary genes and nuclear and cytoplasmic modifying factors is proposed.

1. INTRODUCTION

Polyene resistant mutants of yeast are of interest because of their altered cell membranes (Woods, 1971; Thompson, Starr & Parks, 1971; Bard, 1972; Molzahn & Woods, 1972; Hsuchen & Feingold, 1974) and their possible significance in the medical treatment of yeast infections (Athar & Winner, 1971; Patel & Johnston, 1971; Hamilton-Miller, 1974). Polyene resistant fungal mutants have been isolated, with or without prior mutagen treatment, from *Candida* species (Hamilton-Miller, 1972*b*; Hsuchen & Feingold, 1974; Woods *et al.* 1974), from *Saccharomyces cerevisiae* (Ahmed & Woods, 1967; Patel & Johnston, 1968), from *Saccharomycopsis lipolytica* (Karunakaran & Johnston, 1973), and from *Neurospora crassa* (Grindle, 1973). A number of genes responsible for conferring polyene resistance have been identified in *Saccharomyces cerevisiae* (Ahmed & Woods, 1967; Johnston, 1971; Bard, 1972).

This paper reports on the presence in *Saccharomyces cerevisiae* of both dominant and recessive primary genes that confer resistance to the polyene nystatin and on their allelic and linkage relationships. Data are also presented to show that increased resistance to nystatin can be achieved by the combination of certain primary mutant genes or the combination of specific modifier genes with primary resistance genes. A generalized model is proposed on how mutants achieve secondary levels of resistance.

2. MATERIALS AND METHODS

(i) *Strains*

The following nystatin-resistant mutants of *Saccharomyces cerevisiae* were obtained from the Deparmental Collection (Patel, 1968; Coulson, 1970): M9, N95, N65, P11, N57, N45a, N77 and P29. Another six polyene resistant mutants, *nys1*, *pol1*, *pol2*, *pol3*, *pol4* and *pol5* (Ahmed & Woods, 1967; Molzahn & Woods, 1972), were obtained from Dr R. A. Woods (Sheffield). Laboratory marker strains of *Saccharomyces* were obtained either from Drs J. Bassel and R. K. Mortimer, The Yeast Genetics Stock Center, Berkeley, or had been bred within the Department, having been derived from Carbondale-Berkeley/Seattle stock. The marker strains, with their genotypes in parentheses, were:

X764-S1 (*α*, *arg4*, *his6*, *hom3*, *ura3*)
X764-S2 (*a*, *ade6*, *arg4*, *hom3*, *leu1*, *lys1*)
X901-35C (*a*, *ade6*, *arg4*, *his6*, *hom1*, *leu1*, *lys1*, *thr1*, *trp5*, *tyr4*, *ura1*)
X3104-8C (*a*, *ade2*, *can1*, *his2*, *his6*, *ilv3*, *leu2*, *lys1*, *met14*, *pet8*, *tyr7*, *uvs9*)
X3382-3A (*a*, *ade1*, *arg4*, *asp5*, *cdc14*, *gal1*, *his2*, *his6*, *leu1*, *pet17*, *trp1*, *tyr7*)
S732C (*a*, *ade1*)
S1780B (*a*, *ade1*, *gal1*, *lys1*, *thr1*, *ura2*, *ura4*)

The gene symbols *ade*, *arg*, *asp*, *his*, *hom*, *ilv*, *leu*, *lys*, *met*, *thr*, *trp*, *tyr*, *ura* denote requirement for, respectively, adenine, arginine, aspartate, histidine, homoserine (or methionine + threonine), isoleucine + valine, leucine, lysine, methionine, threonine, tryptophan, tyrosine + phenylalanine, uracil; *gal* denotes non-fermentation of galactose; *pet* denotes respiratory deficiency (petite); *can* denotes canavanine-resistance; *uvs* denotes ultraviolet sensitivity; *cdc* denotes late nuclear division (temperature-sensitive lethal).

(ii) *Media*

Most media for culture maintenance, growth, fermentation and sporulation were as described by Mortimer & Hawthorne (1969).

Nystatin stock solution, of concentration 10000 units/ml (u/ml), was freshly made when required by dissolving sterile nystatin powder (E. R. Squibb & Sons) in dimethyl sulphoxide (BDH Chemicals Ltd). Appropriate volumes were added to cooled MYGP medium (50 °C), plates immediately poured, and these were used within a few hours of preparation.

(iii) *Procedures*

Routine methods for growth, hybridization and sporulation were those described by Mortimer & Hawthorne (1969).

When testing strains for nystatin resistance, the strains were streaked or replica-plated on to MYGP medium supplemented with varying concentrations of nystatin and the plates were scored for growth or non-growth after 72 h at 30 °C. All levels of resistance indicated in text are in units nystatin/ml. All nystatin-resistant

mutants were maintained in complete medium and stored at room temperature (Karunakaran & Johnston, 1974).

3. RESULTS

(i) *Primary analysis of mutants*

The nystatin-resistant mutants M9-K1, N95-K1 and *nys1*-K1 were isolated from the mutants M9, N95 and *nys1* respectively by recurrent culturing of these mutants in increasing concentrations of nystatin in MYGP broth. On solid medium, M9-K1 and N95-K1 were both resistant to 200 u/ml whilst *nys1*-K1 was resistant to

Table 1. *Resistance levels of mutants examined for further genetic analysis*

Mutants	Resistance (u/ml)
M9-K1, N95-K1	200
nys1-K1, K2	150
N65	80
K1, P11, N57	60
N45a	40
pol1, pol2	30
N77, P29, *pol3*	10–20
pol4, *pol5*	5–10

Table 2. *Tetrad analysis of crosses of resistant (R) mutants × sensitive (S) strains showing segregation of single primary resistance genes*

Mutant	Number of tetrads	Segregation ratios of tetrads
N95-K1	69	68 (2R:2S); 1 (3R:1S)
M9-K1	73	70 (2R:2S); 2 (3R:1S) 1 (1R:3S)
nys1-K1	60	60 (2R:2S)
K1	19	19 (2R:2S)
N45a	16	16 (2R:2S)
K2	33	32 (2R:2S); 1 (3R:1S)
pol1	8	8 (2R:2S)
pol2	11	9 (2R:2S)

150 u/ml. Mutant K1 was an isolate from strain S732C and showed resistance to 60 u/ml. The more highly resistant mutant (150 u/ml) K2, was isolated from a resistant segregant (40 u/ml) obtained from the cross M9-K1 × S732C. Table 1 lists the nystatin-resistant mutants examined and their respective levels of resistance. Mutants that showed a level of resistance below 20 u/ml, i.e. N77, P29, *pol3*, *pol4* and *pol5*, were difficult to distinguish from sensitive strains and were excluded from further analysis.

Tetrad analysis was used to elucidate the genetic background of the mutants. Table 2 shows the pattern of tetrad segregations of crosses of sensitive strains with nystatin-resistant mutants evidently carrying single primary genes for resistance. The resistant segregants from the cross N95-K1 × S732C showed levels of resistance ranging from 30–150 u/ml. Low resistance segregants (30–60 u/ml), when crossed

with sensitive strains, showed segregation for a single gene conferring resistance at 40 ± 10 u/ml. Segregants with higher resistance, when crossed with sensitive strains, gave resistant segregants showing levels of resistance ranging from 30 u/ml to that of the resistant parent used. These results can be explained by proposing that the primary gene is responsible for conferring resistance at 40 ± 10 u/m, and that the higher levels of resistance are due to secondary genes and/or extrachromosomal factors. The primary gene in mutant N95-K1, designated *NYSA*, showed dominance as diploid crosses were resistant to 40 u/ml.

Similarly, mutants *nys1*-K1 and M9-K1 both showed the presence of primary genes, designated respectively *nysB* and *NTSD*, each conferring resistance of 40 ± 10 u/ml. Both mutants also appeared to carry secondary factors that increased their resistance to 150 u/ml and 200 u/ml respectively. Both K1 and N45a had only primary resistance genes, designated respectively *NYSE* (50 ± 10 u/ml) and *nysC* (40 ± 10 u/ml). The genes *NYSD* and *NYSE* showed dominance while mutations *nysB* and *nysC* were recessive.

The cross K2 × S732C and most of its resistant segregants showed levels of resistance at 140 ± 10 u/ml although a few segregants (3 out of 26) were resistant to 80 ± 10 u/ml. These latter segregants (80 ± 10 u/ml), when crossed with sensitive strains, showed segregation for a single dominant gene that conferred resistance to 80 ± 10 u/ml. However, the more highly resistant segregants (140 ± 10 u/ml), when crossed with sensitive strains, always produced segregants resistant either to 140 ± 10 u/ml or to 80 ± 10 u/ml level. It was also observed that some of the segregants of higher resistance (140 ± 10 u/ml) were unstable at this level and tended to revert to the lower level (80 ± 10 u/ml). Thus mutant K2 was postulated to have a dominant primary gene, designated *NYSF*, that confers resistance to 80 ± 10 u/ml, and, in addition, either a closely linked dominant modifier gene or an extrachromosomal factor that increases the level of resistance to 140 ± 10 u/ml in the presence of *NYSF*. Unfortunately, the instability of this secondary factor did not allow its isolation.

Very few of the segregants from the crosses of mutants N57, N65 and P11 with sensitive strains were resistant. Cross N57 × X764-S1 gave 3 resistant segregants (2 at 20 u/ml and 1 at 40 u/ml) out of a total of 52 segregants. Cross N65 × X764-S1 gave 3 resistant segregants (all resistant to 20 u/ml) out of a total of 140 segregants. Only 1 of the 80 segregants from the cross P11 × S732C was resistant (20 u/ml). However, the three mutants, N57, N65 and P11, were all very stable with respect to resistance and during the course of analysis none of them reverted to sensitivity. The high proportion of sensitive segregants obtained from each of the three crosses indicates that resistance in these mutants is probably due to non-nuclear factors. The mutants N57, N65 and P11 were tentatively classified as cytoplasmic mutants.

(ii) *Analysis for allelism between primary genes*

Tests for allelism between the primary genes carried by mutants N95-K1 (*NYSA*), *nys1*-K1 (*nysB*), N45a (*nysC*), M9-K1 (*NYSD*), K1 (*NYSE*), and K2 (*NYSF*) were carried out by analysing the segregation patterns of the tetrads

from crosses between mutants (Table 3). The gene *NYSA* was observed to be non-allelic with each of the genes, *nysB* (Table 3*a*), *nysC* (Table 3*b*), *NYSD* (Table 3*c*), and *NYSF* (Table 3*e*). However, *NYSA* is apparently allelic with *NYSE*. Alternatively, if they are separate genes, they are very closely linked (Table 3*d*). The gene *nysB* was observed to be non-allelic with the genes *NYSD* (Table 3*g*) and *NYSF* (Table 3*i*). However, *nysB* seems allelic (or very closely linked) with the genes *nysC* (Table 3*f*) and *NYSE* (Table 3*h*). The genes *NYSE* and *NYSF* were observed to be separate genes (Table 3*m*). The data obtained also indicate *NYSD* to be non-allelic with *nysC* (Table 3*j*), *NYSE* (Table 3*k*), and *NYSF* (Table 3*l*).

Table 3. *Tetrad analysis of crosses between mutants indicating possible allelic and linkage relationships*

	Cross	Allelic (A) or non-allelic (NA)	PD:NPD:T ratio	Linked (L) or non-linked (NL)	Map distance (cM)
a	*NYSA* × *nysB*	NA	204:0:6	L	1·4
b	*NYSA* × *nysC*	NA	86:0:17	L	8·3
c	*NYSA* × *NYSD*	NA	37:7:32	L (?)	> 50·0
d	*NYSA* × *NYSE*	A (?)	120:0:0	L (if NA)	< 1·0
e	*NYSA* × *NYSF*	NA	9:1:29	L (?)	44·9
f	*nysB* × *nysC*	A(?)	120:0:0	L (if NA)	< 1·0
g	*nysB* × *NYSD*	NA	10:10:37	NL	.
h	*nysB* × *NYSE*	A (?)	132:0:0	L (if NA)	< 1·0
i	*nysB* × *NYSF*	NA	17:12:41	NL	.
j	*nysC* × *NYSD*	NA	15:24:56	NL	.
k	*NYSD* × *NYSE*	NA	23:15:33	NL	.
l	*NYSD* × *NYSF*	NA	59:3:21	L	23·5
m	*NYSE* × *NYSF*	NA	16:19:41	NL	.

It is clear from these results that *NYSD* and *NYSF* are separate genes and each is non-allelic to any of the genes *NYSA*, *nysB*, *nysC*, or *NYSE*. However, the results do not unambiguously elucidate the relationships between the genes *NYSA*, *nysB*, *nysC* and *NYSE*. The possible allelic and linkage relationships between these genes are summarized in Table 3. Different interpretations as to the relationships between *NYSA*, *nysB*, *nysC* and *NYSE* are, however, possible. The data may be taken to indicate that *NYSA* and *nysB* are very closely linked and separated by a map distance of 1·4 cM (Table 3*a*), whilst *NYSA* and *nysC* are less closely linked and separated by 8·3 cM (Table 3*b*). However, *nysB* and *nysC*, which are both recessive genes, appear to be allelic since *nysB* × *nysC* diploids were resistant and since no sensitive segregants were detected in the 120 tetrads analysed from this cross (Table 3*f*).

The results obtained from the crosses between the genes *NYSA*, *nysB* and *nysC* can be explained if these genes are assumed to be separate genes which are linked in the order shown in Figure 1. The apparent allelism between *nysB* and *nysC* can be accounted for if mutant *nys1*-K1 carries a deletion covering gene *nysB* and partially overlapping the gene *nysC*, i.e. this mutant would be

genotypically classified as *nysB nysC*. In this case, the diploid obtained from the cross *nysB* × *nysC* would be effectively homozygous for *nysC* and therefore resistant. This would also account for the map distance of 1·4 cM between *NYSA* and *nysB* (Table 3*a*), 8·3 cM between *NYSA* and *nysC* (Table 3*b*), and < 1·0 cM between *nysB* and *nysC* (Table 3*f*).

The mutation *NYSE* seems to be allelic with *NYSA* (Table 3*d*) and *nysB* (Table 3*h*). If *NYSA* and *nysB* are separate genes, then *NYSE* can either be allelic to *NYSA*, since both these genes are dominant, or it could be a separate gene between *NYSA* and *nysB*. However, if *NYSE* is allelic to *NYSA*, then several sensitive segregants should be detected among the 132 tetrads of the *nysB* × *NYSE* cross; no sensitive segregants were detected in this cross (Table 3*h*).

Genes *NYSD* and *NYSF* are non-allelic and have a map distance of 23·5 cM between them (Table 3*l*). Gene *NYSF* may be linked to *NYSA* with 44·9 cM between them (Table 3*e*). However, the total number of tetrads analysed in the *NYSA* × *NYSF* cross was only 39, because of poor spore viability. Though the apparent linkage between *NYSA* and *NYSF* is not conclusive, the data obtained from the *NYSD* × *NYSA* cross supports the presence of linkage (Table 3*c*). If these three genes, *NYSA*, *NYSD* and *NYSF*, are linked, then *NYSF* lies between *NYSD* and *NYSA*. Gene *NYSD* shows no linkage with either *NYSE* (Table 3*k*) or with *nysC* (Table 3*j*). Gene *NYSF* and *NYSE* also show lack of linkage (Table 3*m*). Thus the gene from the cluster, *NYSA*, *NYSE*, *nysB*, *nysC*, closest to *NYSF* seems to be *NYSA*.

(iii) *Positive interaction between primary genes*

When two mutants carrying non-allelic primary genes for resistance are crossed, one of the three resistant segregants from tetratype (T) tetrads and both the resistant segregants from non-parental ditype (NPD) tetrads will inherit both primary genes for resistance. All other resistant segregants, the other two from T tetrads and the four from parental ditype (PD) tetrads, will inherit only one of the two primary genes for resistance carried by the parental strains.

On analysing tetrads from the crosses *NYSD* × *nysB*, *NYSD* × *nysC* and *NYSD* × *NYSE*, it was observed that some of the resistant segregants showed levels of resistance higher than either of the parental strains; generally, both the resistant segregants from NPD tetrads and one of the three resistant segregants from T tetrads were those that showed increased levels of resistance (Table 4). These observations showed that increased levels of resistance were obtained when the gene *NYSD* was in combination with one of the genes *nysB*, *nysC* or *NYSE*. The increased levels of resistance were approximately equal to the additive levels of resistance of the parents. The cases where additivity is expected but not observed could be due to gene conversion or lack of penetrance of either of the genes involved. Gene *NYSD* in combination with either of the other genes, *NYSA* or *NYSF*, did not show any additive effects. To test whether the additivity could be increased further by *NYSD* in combination with two of the three genes *nysB*, *nysC* and *NYSE*, crosses of *NYSD*, *nysC* × *nysB* and *NYSD*, *nysC* × *NYSE* were analysed.

All segregants from 29 tetrads of the *NYSD*, *nysC* × *nysB* cross and from 10 tetrads of the *NYSD*, *nysC* × *NYSE* cross were resistant. This result suggests that each segregant from these tetrads had at least one resistant gene precluding the possibility of any one segregant inheriting all three parental resistant genes. This result is due to the close linkage relationships between the genes *nysB*, *nysC* and *NYSE* (Fig. 1). A very large sample of tetrads would need to be analysed to

Table 4. *Positive interaction between the gene* NYSD *and each of the genes* nysB, nysC *and* NYSE

Cross (parent resistance, u/ml)	Types of tetrads (PD, NPD or T)	Number of tetrads in which segregants (0, 1 or 2) showed an additive level of resistance (90 ± 20 u/ml) 0	1	2
NYSD × *nysB* (40 ± 10)	10 PD	10	0	0
	10 NPD	1	2	7
	37 T	2	30	5
NYSD × *nysC* (40 ± 10)	15 PD	15	0	0
	24 NPD	0	1	23
	56 T	3	49	4
NYSD × *NYSE* (40–50 ± 10)	23 PD	18	5	0
	15 NPD	0	8	7
	33 T	4	29	0

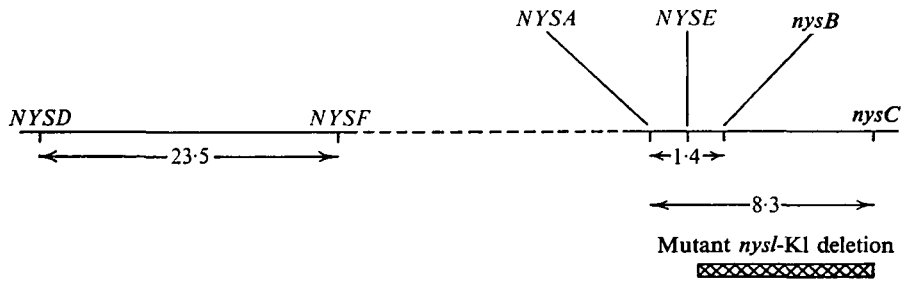

Fig. 1. The proposed genetic map of nystatin-resistant genes. The map distances are given in centimorgans (cM).

obtain a few segregants carrying either both *nysB* and *nysC* or both *nysC* and *NYSE*. However, it has been proposed that the mutant carrying *nysB* is carrying also *nysC* and this mutant has the same level of resistance as the mutant carrying only gene *nysC*.

(iv) *Linkage analysis*

It was decided to attempt to map *NYSA* and *NYSD* since the linkage of these two genes with the genes *nysB*, *nysC*, *NYSE* and *NYSF* (Fig. 1) means that all six genes can be assigned to a particular chromosome. However, in a large number of crosses made, poor viability of the ascospore progeny restricted the attempts to map these two genes.

The data obtained from some of the crosses, however, show that *NYSA* is

centromere-linked whilst *NYSD* is not (Table 5). Crosses of *NYSA* with centromere-linked genes, *ade1*, *leu1*, *leu2* and *his2*, gave PD:NPD:T ratios of 1:1:<4. Since the PD:NPD ratios in these crosses were approximately 1:1, *NYSA* is not linked to any of these genes and is therefore probably not on chromosome I, III, VI or VII. When *NYSD* was crossed to centromere-linked markers, *ade1* and *leu2*, the PD:NPD:T ratios were approximately 1:1:4, indicating that *NYSD* was not centromere-linked. Gene *NYSD* was also shown unlikely to be linked to the non-centromere marker *ura1*, which is on chromosome XI.

Table 5. *Tetrad analysis of crosses of nystatin-resistant mutants with marker strains*

Cross	Marker gene centromere linked (+) non-centromere linked (−)	Chromosome number	PD	NPD	T
NYSA × *ade1*	+	I	20	22	12
NYSA × *leu2*	+	III	12	15	13
NYSA × *his2*	+	VI	12	12	20
NYSA × *leu1*	+	VII	16	19	7
NYSA × *ura1*	−	XI	11	12	40
NYSD × *ade1*	+	I	8	9	44
NYSD × *leu2*	+	III	12	7	37
NYSD × *ura1*	−	XI	1	3	4

Table 6. *Random spore analysis of crosses of mutant* NYSA *with centromere marker strains*

Marker gene	Chromosome number	Type of spore: Parental	Type of spore: Recombinant
trp1	IV	27	25
arg4	VIII	29	23
asp5	VIII	29	23
tyr7	XVI	29	23

It was decided at this stage to attempt to map *NYSA* since the existence of multi-marker strains (X3104-8C, X3382-3A) makes it easier to map centromere-linked genes compared to non-centromere-linked genes. If *NYSA* could be assigned to a particular chromosome, then the other nystatin-resistant genes, including *NYSD*, could be checked for location on the same chromosome. The data, obtained by random spore analysis (Table 6), indicate that *NYSA* was not on chromosome IV, VIII or XVI. Thus *NYSA* is apparently located on a chromosome other than I, III, IV, VI, VII, VIII and XVI.

(v) *Secondary levels of resistance*

When the nystatin-resistant mutants, N95-K1, M9-K1 and *nys1*-K1, were crossed with sensitive strains, a wide range of resistance levels, 30–150 u/ml, was

obtained in the segregants. A systematic analysis was carried out with the mutant N95-K1 to attempt to isolate factors responsible for conferring different levels of resistance. A sample of resistant segregants from the N95-K1 × S732C cross, representative of the range of resistance levels, were crossed with sensitive strains and ascospore progeny tested (Table 7). The results show that the levels of resistance of the progeny were, at most, equal to that of the resistant parent in the particular cross. When resistant segregants showing resistances at 40 u/ml were crossed with sensitive strains, all resistant progeny obtained showed levels of resistance at 40 ± 10 u/ml, indicating the presence of only gene *NYSA*. However, crosses of more highly resistant segregants with sensitive strains produced progeny with resistance levels ranging from 40 ± 10 u/ml to the level of the resistant parent

Table 7. *Analysis of crosses of sensitive strains with resistant segregants from cross N95-K1 × S732C*

(Level of resistance in u/ml given in parentheses.)

	Cross	Resistance level of diploid	Number and resistance of segregants
a	XK2-12C (150) × X764-S1 (0)	40	14 (0); 3 (40); 1 (60); 3 (80); 2 (100); 5 (150)
b	XK2-2C (80) × X764-S1 (0)	30	18 (0); 3 (30); 2 (40); 5 (60); 8 (80)
c	XK2-4C (60) × S732C (0)	40	31 (0); 2 (30); 13 (40); 18 (60)
d	XK2-9B (40) × S732C (0)	40	12 (0); 4 (30); 6 (40)

in the cross. These results indicate that a segregant from the N95-K1 × S732C cross with resistance higher than 40 u/ml carries the primary gene *NYSA* and one or more secondary factors that in the presence of the primary gene modify the level of resistance.

Segregants from the N95-K1 × S732C cross were backcrossed to N95-K1 and the progeny analysed for resistance levels (Table 8). When sensitive segregants were backcrossed to N95-K1, segregants showing a wide range of resistance, 20–180 u/ml, were obtained (Table 8*a*, *b*). The range of resistance obtained was wider, but only by a narrow margin, than that obtained from the cross N95-K1 × S732C. This would be expected if the sensitive segregants from the N95-K1 × S732C cross carried some secondary factors responsible for increasing resistance, but only in the presence of the primary gene *NYSA* or in combination with *NYSA* and other secondary factors. Segregants carrying gene *NYSA* and more secondary factors would thus be expected to show a level of resistance closer to that of N95-K1, whilst those carrying gene *NYSA* and no secondary factors would be resistant to 40 ± 10 u/ml (the primary gene level).

When more highly resistant segregants from the N95-K1 × S732C cross were backcrossed to N95-K1, some of the progeny obtained were resistant to 200 u/ml (Table 8*c* and 8*d*). However, a large number of resistant segregants from these backcrosses showed levels of resistance below that of the lesser resistant parent. This indicates that not only the presence of secondary factors is required for higher resistance but that particular combinations of these factors determines the level of resistance expressed by the segregant. Sensitive segregants and highly resistant segregants from the cross N95-K1 × S732C were crossed to try to obtain a segregant

Table 8. *Backcrosses of segregants from the cross N95-K1 × S732C with the resistant parent*

(Level of resistance in u/ml given in parentheses.)

	Cross	Resistance level of diploid	Number and type of tetrads	Number and resistance of segregants
a	XK2-9D (0) × N95-K1 (200)	60	6 (2S:2R)	12 (0); 2 (30); 2 (40); 3 (80); 2 (100); 3 (150)
			5 (1S:3R)	5 (0); 1 (30); 4 (40); 3 (60); 1 (80); 2 (100); 4 (150)
b	XK2-3A (0) × N95-K1 (200)	80	8 (2S:2R)	16 (0); 2 (30); 1 (40); 3 (60); 6 (80); 3 (150); 1 (180)
			4 (1S:3R)	4 (0); 1 (20); 3 (30); 1 (60); 4 (80); 1 (100); 2 (150)
c	XK2-8A (100) × N95-K1 (200)	60	5 (0S:4R)	5 (30); 3 (60); 4 (100); 1 (130); 3 (150); 4 (200)
d	XK2-12C (150) × N95-K1 (200)	40	6 (0S:4R)	1 (20); 2 (30); 7 (40); 2 (60); 1 (80); 4 (100); 2 (130); 1 (150); 4 (200)

showing resistance as high as that expressed by N95-K1 (200 u/ml). One segregant out of a total of 118 resistant segregants analysed showed a level of resistance of 180 u/ml, but none were resistant to 200 u/ml. This result suggests that the number of factors involved in producing secondary levels of resistance is many and that each is responsible for only a small step increase. Thus to obtain a segregant with the same resistance level as N95-K1, a large number of crosses and their progeny would have to be tested. The tetrads obtained from the backcrosses of sensitive segregants with N95-K1 showed a high proportion of 1S:3R ratios, 9 out of 23 tetrads (Table 8*a*, *b*). One possible explanation for this result is the presence of *two* primary genes for resistance in N95-K1, one being *NYSA*. However, if this had been the case, the second primary gene should have been detected in cross N95-K1 × S732C and some resistant progeny from this cross. The data obtained do not therefore indicate the presence of a second primary gene for resistance in N95-K1. Another possible explanation for the 1S:3R tetrads is that certain combinations of secondary factors may also be responsible for *low* levels of resistance scored as 'sensitive'. The probability of these combinations being present is greater

in progeny from backcrosses than in progeny from crosses of resistant mutants with sensitive strains.

The hypothesis that secondary factors contribute small step increases in resistance was strengthened by the isolation of a modifier gene, designated *mod*. A segregant from the cross N95-K1 × S732C, XK2-4C, resistant to 60 u/ml was crossed to S732C. Tetrads of this cross gave three types of segregants: sensitive, resistant to 30–40 u/ml, and resistant to 50–60 u/ml. Diploid cells of cross XK2-4C × S732C were resistant to 40 u/ml. The hypothesis that a recessive modifier gene, *mod*, was responsible for the increase in resistance from 30–40 to 50–60 u/ml, in the presence of the primary gene *NYSA* was tested. Accordingly, the sensitive segregants of a (0, 0, 50–60, 50–60) tetrad from the above cross would have the genotype *NYSA*$^+$, *mod*$^+$ whilst the genotype of the two resistant segregants would be *NYSA*, *mod*. The sensitive segregants of a (0, 0, 30–40, 30–40) tetrad would be *NYSA*$^+$, *mod* whilst the resistant segregants would be *NYSA*, *mod*$^+$.

Table 9. *Analysis of crosses testing for the presence of modifier gene, mod*

(L = 30–40 units/ml and H = 50–60 units/ml.)

	Cross, proposed genotype	Types of tetrads expected	Number and types of tetrads obtained
a	*NYSA*, *mod*$^+$ (L)	(O, O, L, L)	7 (O, O, L, L)
	×	(O, O, L, H)	15 (O, O, L, H)
	NYSA$^+$, *mod* (O)	(O, O, H, H)	6 (O, O, H, H)
b	*NYSA*, *mod* (H)	(H, H, H, H)	11 (H, H, H, H)
	×		
	NYSA, *mod* (H)	—	—
c	*NYSA*, *mod*$^+$ (L)	(L, L, L, L)	6 (L, L, L, L)
	×		
	NYSA, *mod*$^+$ (L)	—	—
d	*NYSA*, *mod* (H)	(L, L, H, H)	13 (L, L, H, H)
	×		3 (L, L, L, H)
	NYSA, *mod*$^+$ (L)	–	1 (L, H, H, H)

The expected and observed results of crosses analysed to test the modifier hypothesis are compared in Table 9. The results substantially verify the hypothesis that the modifier gene, *mod*, is responsible for an increase in resistance, from 30–40 to 50–60 u/ml, in the presence of the primary gene *NYSA*. The aberrant tetrads obtained in the cross *NYSA*, *mod* × *NYSA*, *mod*$^+$ (Table 9*d*) could have been due to conversion and/or the instability of the modifier gene.

Another type of interaction and modification, namely suppression of secondary levels of resistance of N95-K1, was seen when N95-K1 was crossed with the mutant N57; the ascospore progeny analysed showed no segregant having a resistance higher than 40 u/ml (Table 10*a*). However, when mutant N57 was crossed to the mutant *nys1*-K1, there was no detectable suppression of secondary levels of resistance (Table 10*b*). When the ascospore progeny from crosses N57 × *pol1* and *N57* × *pol2* were checked for resistance levels, the crosses gave segregants ranging in

resistance from 20–40 u/ml (Table 10*c*, *d*). Thus the suppressive action of N57 on secondary factors responsible for higher resistance levels in N95-K1 seems to be a specific effect. This suppressive factor or factors could independently suppress the expression of several nuclear genes and/or extrachromosomal elements inherited from N95-K1 and responsible for secondary levels of resistance. Alternatively, a more likely mode of suppression is the effect of the suppressive factor on a specific genetic element responsible for initiating the secondary levels of resistance of N95-K1. One possibility for the latter mechanism is that high resistance of N95-K1 is mediated in stepwise increases (as substantiated by results), that the initial increase is dependent on the presence of the primary gene, *NYSA*, and that further secondary increases are dependent on the presence of the factor responsible for the immediate prior increase. Thus the manner of modification, at least in the case of the *NYSA* gene, may be sequential and cascade-like.

Table 10. *Types of segregants obtained from crosses of proposed cytoplasmic mutant, N57, with nuclear gene mutants*

(Levels of resistance in parentheses.)

	Cross	Resistance levels (u/ml) of parent 1, parent 2, diploid	Number and resistance (u/ml) of different types of tetrads or random segregants
a	N57 × N95-K1	60, 200, 40	4 (0, 0, 30, 30) 6 (0, 0, 30, 40) 8 (0, 0, 40, 40)
b	N57 × *nys1*-K1	60, 150, 30	6 (150), 2 (100), 5 (80), 6 (60), 1 (40), 7(30), 11 (0)
c	N57 × *pol1*	60, 30, 0	13 (40), 5 (30), 6(20), 12 (0)
d	N57 × *pol2*	60, 30, 0	15 (40), 3 (30), 4 (20), 13 (0)

4. DISCUSSION

The foregoing results have produced further evidence that a basic level of resistance to nystatin in most mutants of yeast is due to gene mutations. Primary genes, both dominant and recessive, which are responsible for conferring resistance to nystatin have been identified. These have been designated as *NYSA*, *NYSD*, *NYSE*, *NYSF* (dominant), *nysB* and *nysC* (recessive). The level of resistance conferred by these primary genes varies from 30 u/ml to the relatively high level of 80 u/ml. The primary genes identified by Ahmed & Woods (1967), *nys1*, *nys2* and *nys3*, were recessive and all conferred relatively low levels of resistance, 10–20 u/ml. The resistance levels of two further recessive genes, *pol4* and *pol5*, reported by Molzahn & Woods (1972) were also around 20 u/ml. However, these levels of resistance are not directly comparable to those of the genes described in this paper since the preparation of nystatin medium is different in the two cases. The nystatin-resistant mutants isolated by Bard (1972), *nyr1*, *nyr2*, *nyr5*, *nyr6* and

nyr15, were all recessive single gene mutants that showed a wide range of resistance levels, 50–800 u/ml; however, the high level of resistance is due to Tween 80 in the medium and also the analysis does not exclude the possibility that secondary factors for resistance were present in these mutants.

Two modifier genes were demonstrated to exist in the mutants analysed; one was dominant, linked to *NYSF*, and enhanced the resistance level conferred by *NYSF* from 80 to 140 u/ml; the other, *mod*, was recessive and was responsible for enhancing the resistance conferred by *NYSA* from 40 to 60 u/ml. Two dominant modifier genes, both responsible for relatively small increases in resistance, 5–10 u/ml, were isolated by Ahmed & Woods (1967) and they also showed the effect of these genes to be locus-specific. The specificity of modifier gene action has also been demonstrated in the case of actidione resistance in *S. cerevisiae* (Wilkie & Lee, 1965). Ahmed & Woods (1967) further demonstrated that certain combinations of modifiers with primary mutant genes can be responsible for a second-step secondary increase in resistance. A similar proposal of small stepwise increases in resistance is advanced to explain certain of the results described in this paper.

The mode of action of some modifier genes affecting resistance may be indirect. For example, the clumping of yeast cells may increase the tolerance limit to polyene antibiotics by producing a shielding effect (R. A. Woods, personal communication). Some modifier genes may therefore be simply mutations that cause clumping or flocculation of the cells (Farris & Gilmore, 1974; Lewis, Johnston & Martin, 1976). Mutations that act by altering constituents of the cell membrane other than sterols, for instance the protein and lipid composition, may also have an effect on the available effective binding sites, thus altering the resistance level of the mutant. This indirect action may explain why modifier genes do not, in the absence of primary resistance mutations, themselves confer resistance.

The gene *NYSD* has been shown to give additive levels of resistances in combination with each of the genes *nysB*, *nysC*, or *NYSE*. This represents the initial step for a system of polygenic inheritance as outlined by Darlington & Mather (1949) and Dobzhansky (1955) and as visualized in the case of streptomycin resistance by Demerec (1945, 1948). Thus there is evidence for at least two mechanisms, by modifier genes and by additivity, for obtaining higher levels of resistance in nystatin mutants. Both of these systems have been observed in the case of actidione resistance in yeast (Wilkie & Lee, 1965). These two mechanisms may also themselves combine to produce higher levels of resistance.

We propose that: (*a*) the quantitative effect of various nuclear and extrachromosomal factors and also their various combinations determine the resistance level of a particular mutant, and (*b*) resistance is mediated in a step-wise manner with each increase being dependent on the previous level of resistance, i.e. a cascade effect of primary and secondary factors. These proposals are supported not only by the presence of modifier genes and additive combinations of polygenes but also by the suppressive effect of the presumptive cytoplasmic mutant N57 on the secondary levels of resistance of mutant N95-K1. Further evidence is provided by the analyses of high level multi-step resistant mutants (Karunakaran, 1974).

Analysis of ascospore progeny obtained from crosses of these mutants with sensitive strains showed the presence of primary resistance genes and secondary mutations, thus indicating a polygenic inheritance. It was also found that these multi-step mutants showed instability at the highest levels of resistance, indicating that alternative paths of increase in resistance may be possible through different combinations of factors at these levels.

In biochemical terms, it has been established that alterations in membrane sterols are associated with resistance to polyenes in *Saccharomyces cerevisiae* (Woods, 1971; Molzahn & Woods, 1972; Bard, 1972), in *Candida albicans* (Athar & Winner, 1971; Hamilton-Miller, 1972*a*; Archer & Gale, 1975), in *Candida tropicalis* (Woods *et al.* 1974), and in *Neurospora crassa* (Grindle, 1973, 1974; Morris, Safe & Subden, 1974). Single gene mutations that confer resistance to polyenes show altered sterol contents (Woods, 1971; Molzahn & Woods, 1972; Grindle, 1974). The evidence presently available does not allow one to propose specific models relating sterol content with levels of resistance. Most mutations resulting in polyene resistance are probably genes coding for enzymes in sterol biosynthesis (Kitajima, Sekija & Nozawa, 1976), but genes controlling biosynthesis of membrane, lipids or proteins may also be involved. Elucidation is required of the biochemical variation in sterols, lipids and membrane proteins of the mutants reported in this paper and of the possible functional significance of the close linkage of some of the genes responsible for nystatin resistance.

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