Changes in genetic constitution and sterol composition during growth of nystatin-resistant heterokaryons of *Neurospora crassa*

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SUMMARY

Heterokaryons of *N. crassa* were synthesized from homokaryotic strains differing in sterol composition and sensitivity to the polyene antibiotic nystatin. Mycelia of the nystatin-sensitive strain erg-1^+ contained ergosterol and episterol, and the nystatin-resistant mutant erg-1 contained fecosterol and lichesterol. Mycelia of heterokaryons with different proportions of erg-1^+ : erg-1 nuclei contained various proportions of the four sterols. Ergosterol was the principal sterol in heterokaryons with more than 5 % erg-1^+ nuclei.

Heterokaryons with various proportions of erg-1^+ : erg-1 nuclei were grown for several weeks along tubes of synthetic media. Growth rates were stable on minimal medium and nutritionally supplemented media but nuclear proportions often fluctuated. Growth rates fell sharply on nystatin-supplemented media and there were adaptive increases in proportions of mutant erg-1 nuclei which resulted in selection of nystatin-resistant homokaryotic mycelia.

1. INTRODUCTION

Many fungi have a prominent vegetative phase in their life cycle during which they produce a network of branching hyphæ, the mycelium. Hyphæ of the saprophytic Ascomycete *Neurospora crassa* are septate filaments containing numerous haploid nuclei in cytoplasmic continuity with one another. The nuclei in homokaryotic hyphæ are genetically identical. Heterokaryotic hyphæ contain more than one nuclear genotype and they arise by mutations of nuclei in homokaryons or by hyphal anastomosis of genetically different homokaryons.

Most *N. crassa* heterokaryons containing two different kinds of haploid nuclei maintain their nuclear proportions and rates of growth during growth along tubes of synthetic media (Pittenger & Atwood, 1954, 1956; Pittenger, Kimball & Atwood, 1955). Changes in nuclear proportions may be associated with increases or decreases in growth but they do not result in selection of a viable homokaryotic mycelium (Ryan & Lederberg, 1946; Davis, 1960; Pittenger & Brawner, 1961; Grindle & Pittenger, 1968). Previous attempts to select homokaryons from heterokaryons may have failed because the genotypes of the component nuclei and

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the selection pressure applied to the heterokaryons were unsuitable. For example, the nuclei differed only with respect to their nutritional markers and selection pressure was applied by varying the supply of growth requirements of the homokaryotic components.

This investigation is concerned mainly with heterokaryons whose nuclei carry the mutant gene *erg-1* or its wild type allele *erg-1+*. Homokaryotic *erg-1* strains are deficient in ergosterol and are more resistant than *erg-1+* strains to the polyene antibiotic nystatin. Nystatin was used to apply selection pressure favouring the *erg-1* component of (*erg-1* + *erg-1+*) heterokaryons.

Table 1. *Strains of* Neurospora crassa

Genotype*	Locus; allele or isolation number; linkage group	Phenotype	Rate of growth (mm/24 h)†
leu	*leu-2*; 37501; IVR	Leucine requirement	92·0±4·1
pan	*pan-2*; Y153M96(B3); VIR	Pantothenic acid requirement	97·3±3·5
erg, pan	*erg-1*; UV-1; VR *pan-2*; Y153M96(B3); VIR	Ergosterol deficient and nystatin resistant; pantothenic acid requirement	65·2±2·2

* All strains are mating type *a*; they are not isogenic.
† Linear growth along 500 mm tubes of agar media at 26 °C, mean and standard deviation. Poor growth of *erg,pan* is a feature of *erg-1* strains.

2. MATERIALS AND METHODS

(i) *Strains*

Heterokaryons (*pan-2, leu-2+* + *pan-2+, leu-2*) and (*erg-1, pan-2, leu-2+* + *erg-1+*, *pan-2+, leu-2*) were synthesized from nutritional mutants (Table 1) as described by Pittenger *et al.* (1955); they will be referred to as heterokaryons A (*pan + leu*) and B (*erg, pan + leu*). The heterokaryons can grow on media lacking growth supplements because their nutritional markers are complementary. Heterokaryons A and B were very similar to laboratory wild type strains in morphology, growth rate and conidiation; their vigorous growth and phenotypic stability on MM indicated that the component mutant strains were heterokaryon-compatible.

The antibiotic nystatin binds to ergosterol in cell membranes, causing changes in membrane permeability and leakage of metabolites (Lampen *et al.* 1962). The *pan* and *leu* strains, which carry the normal *erg-1+* gene and synthesize ergosterol, are unable to grow on media containing more than 3 units/ml nystatin; the *erg, pan* strain, which carries the mutant *erg-1* gene and lacks ergosterol, can grow on media containing 10 units/ml nystatin (Grindle, 1973, 1974).

(ii) *Media*

Heterokaryons were grown on synthetic minimal medium, MM (Vogel, 1964) containing 1·5 % (w/v) sucrose as the carbon source, and solidified with 3 % (w/v)

agar. To determine nuclear proportions, conidia were incubated on dishes of sorbose medium (MS), which is MM containing 1% (w/v) sorbose, 0·2% (w/v) sucrose and 2% (w/v) agar; sorbose reduces growth of *N. crassa* (Tatum, Barratt & Cutter, 1949).

Nutritional supplements (0·01 mg/ml calcium pantothenate; 0·2 mg/ml leucine hydrochloride) were added to media before sterilization. Nystatin (supplied as Mycostatin by E. R. Squibb Ltd., Liverpool) was dissolved in propan-1,2-diol and added to flasks of autoclaved media at 50 °C to give a final concentration of 10 units/ml.

(iii) *Analysis of heterokaryons in growth tubes*

Heterokaryons were grown in tubes similar to those described by McDougall & Pittenger (1962). They were constructed from 500 mm lengths of 25 mm diameter Pyrex tubing with a Quickfit FG25 flat-flange joint fused to each end and three or five glass sampling chimneys inserted at intervals of 30–110 mm (Ogden, 1982). The ends of the tubes were sealed with Teflon disks held in position with Quickfit FG25 flat-flange caps and Büchi RSB7 balljoint clamps. Sampling chimneys were sealed with cotton wool, tubes were sterilized in a hot air oven, and 100 ml autoclaved medium was pipetted into each tube.

Inoculated tubes were incubated at 26 °C and the growth front of each heterokaryon was marked every 24 h. Additional tubes were attached with Büchi clamps to allow uninterrupted linear growth of heterokaryons, and short, triple-jointed tubes diverted them into extra 500 mm tubes containing various media (Ogden, 1982). Conidia below sampling chimneys were removed with a special pipette (de Serres, 1962) and analysed for nuclear proportions as described in Section 2(vi).

(iv) *Analysis of heterokaryons in dishes*

Heterokaryons in 150 mm diameter dishes containing 50 ml media were analysed as shown in Fig. 1. Samples were transferred to sterile dishes, allowed to conidiate at room temperature and high humidity for 2–3 days, and nuclear proportions were determined as described in Section 2(vi).

(v) *Sterol analysis*

Flasks of liquid MM were inoculated with conidia, incubated at 26 °C on a rotary shaker at 300 rev/min, and mycelia were harvested after 24–26 h in the log phase of growth. Part of each mycelium was transferred to a dry flask and kept at room temperature for 2–3 days to conidiate for determinations of nuclear proportions. The remaining mycelium was washed with deionized water, and non-saponifiable extracts were analysed by gas–liquid chromatography using 7 foot columns of OV-17, 3% on Diatomite CLQ (100–120 mesh) at 271 °C as described by Grindle & Farrow (1978).

The gas–liquid chromatograms were unsuitable for quantifying the sterols in cell extracts because the peak areas were incompletely resolved (Fig. 2A). A computer
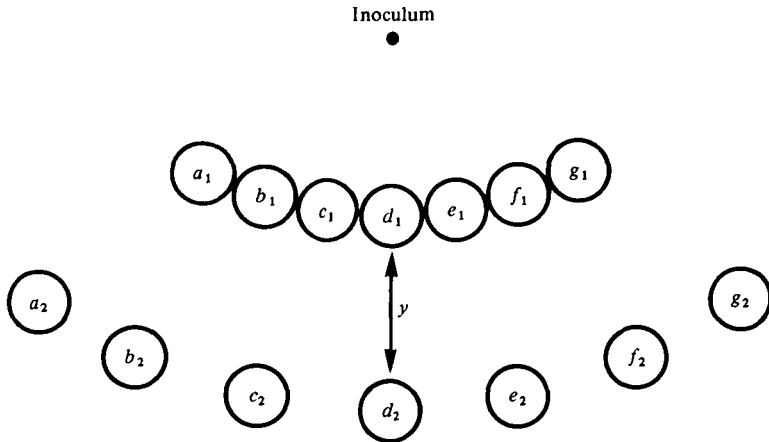
Fig. 1. Method for obtaining samples of mycelia from colonies of heterokaryon B ($erg, pan + leu$). The inoculum is placed at the edge of a dish of agar media, incubated at 26 °C for 15–18 h, and 8 mm diameter samples of mycelia (a_1–g_1) are taken from the colony 10 mm behind the growth front. More samples (a_2–g_2) are taken as before when the growth front has advanced by distance y. Samples 1 and 2 are from the same radii of growth.
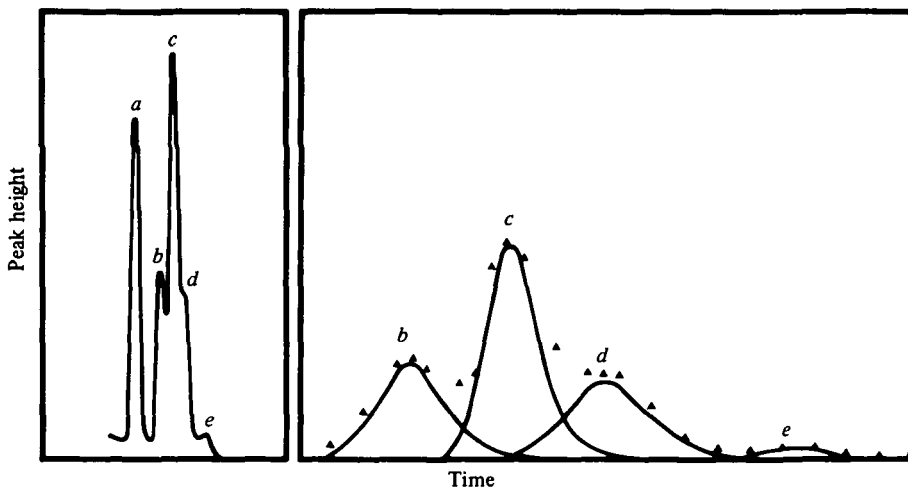

Fig. 2. Gas–liquid chromatography of non-saponifiable extracts of mycelia of a *Neurospora crassa* heterokaryon B containing 90 % erg, pan nuclei. (A) Tracing from original chromatogram, showing the separation of sterols on columns of OV-17. Cholesterol was used to calculate the relative retention time (RRTs) of the other sterols. a, Cholesterol; b, lichesterol (RRT = 1·193); c, ergosterol (RRT = 1·297); d, fecosterol (RRT = 1·373); e, episterol (RRT = 1·524). (B) Computer-derived tracing from the original chromatogram, using the coordinates ▲.

program based on a least-squares analysis (Fraser & Suzuki, 1973) was used to fit the best curves to the original data (Fig. 2B). The sterols were quantified by photocopying the computer-derived tracings on uniform paper and weighing the individual sterol peak areas, and the relative proportions of the sterols in each extract were calculated from these weights.

(vi) *Determination of nuclear proportions*

The relative proportions of the two kinds of nuclei in heterokaryotic mycelia were determined as described by Atwood & Mukai (1955) using the formula $P = [r(1-r) + a(n-2r)] \div n(1-r)$. P is the proportion of type X nuclei in a heterokaryon containing X and Y nuclei; r is the proportion of heterokaryotic conidia; a is the proportion of homokaryotic conidia containing only type X nuclei; n is the mean number of nuclei per conidium. The value for n was determined by staining conidia with Azure A (Huebschman, 1952) and counting the number of nuclei in 200 conidia from two cultures of heterokaryon B; the mean n value of 3·7 was used for all calculations. Values for nuclear proportions will be given as percentages; for example, a P value of 0·2384 will be given as 23·8 % or 24 %.

The proportions of homokaryotic (a) and heterokaryotic (r) conidia were determined as described by Pittenger (1964). Conidia were suspended in 1 % saline, aliquots ($2-3 \times 10^3$) were incubated at 26 °C on dishes of MS and MS with nutritional supplements, and the homokaryotic and heterokaryotic colonies were counted after 2–3 days growth.

Values for a and r are subject to sampling and plating errors. The effects of these errors on calculated nuclear proportions were assessed by analysing three conidial samples from each of the five sampling chimneys of a tube of heterokaryon B to obtain 15 P values for an analysis of variance, and the within-sample root mean square was used as an estimate of the standard deviation of any calculated P value. The overall mean P values of the 15 samples in three separate experiments were 23·8 %, 45·8 % and 89·2 % *erg, pan* nuclei and the pooled standard deviation was ±3·7 % with 30 degrees of freedom.

Similar errors were expected when determining the nuclear proportions of mycelia used for sterol analysis. To estimate these errors, the mycelia from three liquid cultures were each divided into four samples, allowed to conidiate in dry flasks and analysed independently to determine nuclear proportions. The overall mean P values in three separate experiments were 58·9 %, 84·3 % and 93·6 % *erg, pan* nuclei and the pooled standard deviation was ±4·1 % with 27 D.F.

3. RESULTS

The nutritional markers in heterokaryon B were needed to synthesize stable heterokaryons and to simplify the identification of homokaryotic and heterokaryotic conidial progeny. Heterokaryon A was analysed to assess the effects of these nutritional markers in the absence of the mutant *erg-1* gene.

(i) *Analysis of heterokaryon A*

Three cultures of heterokaryon A, initiated from mycelia with 4 %, 80 % and 83 % *pan* nuclei, were analysed for changes in growth rate and nuclear proportions during 5–22 days growth along tubes of MM. The heterokaryons had similar rates of growth (overall mean = 96·5 ± 3·8 mm/24 h) and their nuclear proportions were stable. Cultures diverted into tubes of MM supplemented with leucine or panto-

thenate maintained the same rates of growth and the same nuclear proportions as the parent cultures on MM.

(ii) *Changes in growth rate and nuclear proportions during growth of heterokaryon B in tubes*

Heterokaryons were grown for several weeks in tubes of MM and MM supplemented with nutrients and nystatin. Changes in growth rate and nuclear proportions have been described by Ogden (1982) and are exemplified in Fig. 3.

Five cultures of heterokaryon B, initiated from mycelia with 34 %, 65 %, 76 %, 77 % and 84 % *erg, pan* nuclei, were grown in tubes of MM for 12–23 days. Their rates of growth (overall mean $= 98{\cdot}9 \pm 4{\cdot}1$ mm/24 h) were not significantly different from those of heterokaryon A, but the nuclear proportions of most cultures seemed to increase and decrease at random (Figs 3 A, C). Heterokaryons diverted into tubes of MM supplemented with pantothenate or leucine had the same growth rate as the parent cultures, and there were similar changes in nuclear proportions (Fig. 3 C).

Heterokaryons diverted into tubes of media containing nystatin grew slowly and erratically (mean growth rate $= 45{\cdot}7 \pm 12{\cdot}4$ mm/24 h), growth fronts were extremely irregular and proportions of *erg, pan* nuclei increased abruptly (Fig. 3 A) or gradually (Figs 3 B, C). One heterokaryon on nystatin medium supplemented with pantothenate (Fig. 3 C) and another on nystatin medium supplemented with pantothenate and leucine became homokaryotic after 26–28 days growth (samples of conidia ($2{\cdot}3$–$5{\cdot}9 \times 10^5$) from three successive chimneys of the growth tubes did not grow on MM or MM supplemented with leucine), and the homokaryotic mycelia that emerged from the heterokaryons had the same growth rate and morphology as the original *erg, pan* parent. The nutritional requirement of *erg, pan* mycelia prevented selection of *erg, pan* homokaryons on MM.

(iii) *Variations in nuclear proportions among regions of mycelia of heterokaryon B colonies on dishes*

Nuclear proportions of heterokaryons in tubes often changed considerably between successive sampling chimneys (Figs. 3 A, C). Colonies on dishes were analysed to determine the extent of variations in nuclear proportions among regions of mycelia only a few mm apart.

The results showed that there can be large differences in nuclear proportions among neighbouring regions of mycelia on the same growth front and on the same radius of growth, despite only minor changes in the overall mean nuclear proportions during growth (Table 2). Nuclear proportions of samples from heterokaryons on nystatin media were less variable than those on MM (Table 2).

(iv) *Sterol analysis of heterokaryon B*

Homokaryotic *leu* mycelia (100 % *erg-1*$^+$ nuclei) contained ergosterol and episterol, and homokaryotic *erg, pan* mycelia (100 % mutant *erg-1* nuclei) accumu-

lated lichesterol and fecosterol (Fig. 4). Heterokaryotic mycelia contained various proportions of ergosterol, episterol, lichesterol and fecosterol (Figs. 2, 4). Increase in proportions of *erg, pan* nuclei was associated with increase in relative proportions of lichesterol and fecosterol and decrease in ergosterol. Ergosterol was the principal
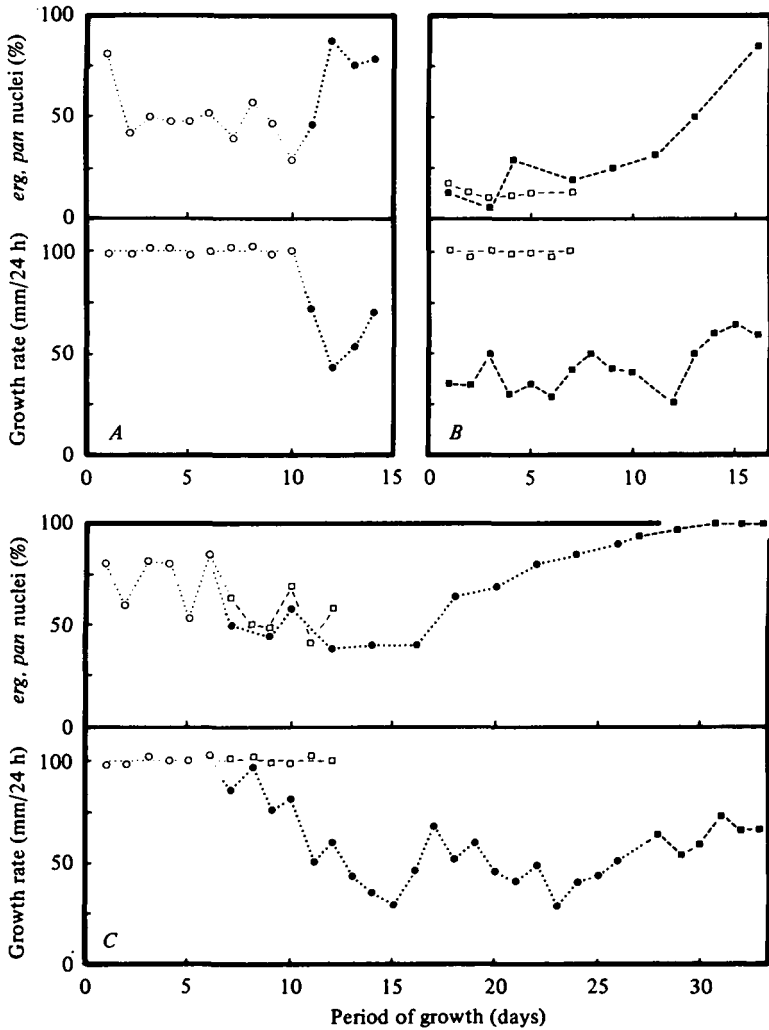

Fig. 3. Changes in growth rate and proportions of *erg,pan* nuclei during growth of *Neurospora crassa* heterokaryons B along tubes of agar media. Minimal medium, MM (○...○); MM plus nystatin (●...●); MM plus pantothenate (□--□); MM plus pantothenate and nystatin (■--■).

sterol of *erg-1*[+] homokaryons and heterokaryons containing more than 5 % *erg-1*[+] nuclei; lichesterol was the principal sterol of mutant *erg-1* homokaryons but it did not exceed 30 % of the sterol content of heterokaryons (Fig. 4).

Extrapolating the data in Fig. 4, there is presumably a rapid decrease in ergosterol content of mycelia and a rapid increase in lichesterol when the

4-2

Table 2. *Proportions of erg,pan nuclei in mycelial samples from colonies of heterokaryon B (erg, pan + leu) on synthetic media*

Medium	Distance y between samples 1 and 2 (mm)*	Samples*							
		a_1 a_2	b_1 b_2	c_1 c_2	d_1 d_2	e_1 e_2	f_1 f_2	g_1 g_2	(Mean 1) (Mean 2)
MM	20	60·8 56·3	48·4 54·5	67·5 44·3	50·6 45·1	50·0 41·8	49·4 45·6	47·8 34·1	(53·5) (46·0)
MM	20	81·8 68·4	68·4 68·1	76·9 73·1	53·4 84·1	68·5 66·3	80·7 63·4	— —	(71·5) (70·6)
MM	15	67·6 56·3	82·2 74·8	85·2 55·2	80·5 76·7	79·4 77·9	78·3 80·3	— —	(78·9) (70·2)
MM + nystatin	55	89·8 91·7	85·0 92·7	90·4 92·4	93·2 94·3	— —	— —	— —	(89·6) (92·8)
MM + nystatin	20	97·9 89·2	98·4 91·6	95·0 95·2	94·0 97·6	92·2 93·2	— —	— —	(95·5) (93·4)

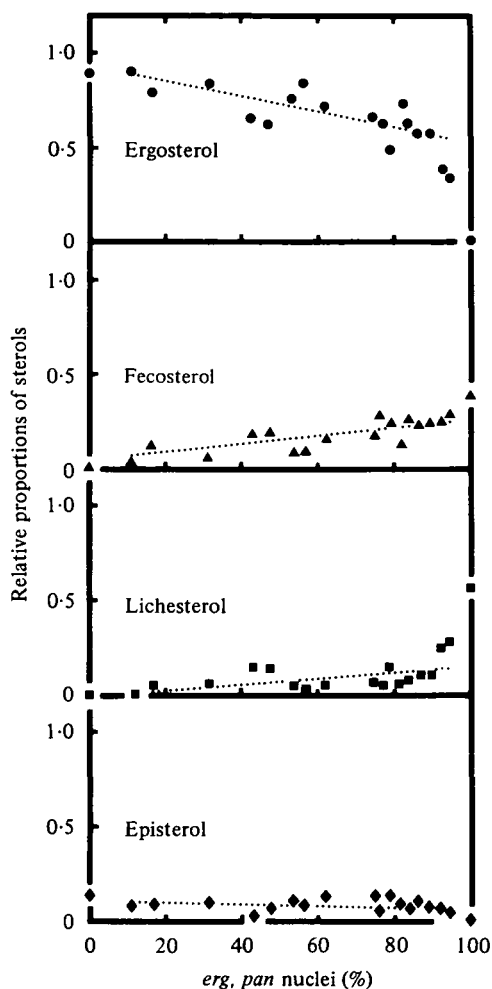* Samples were obtained as shown in Figure 1.   — not tested.

Fig. 4. Relative proportions of sterols in mycelia of *Neurospora crassa* heterokaryons B containing various proportions of *erg,pan* and *leu* nuclei. *erg,pan* nuclei carry the mutant gene *erg-1*, and *leu* nuclei carry the normal gene *erg-1+*. Data for 0 % *erg,pan* relate to *leu* homokaryons, and data for 100 % *erg,pan* relate to *erg,pan* homokaryons. Regression lines were fitted by least-squares analysis.

proportion of *erg-1+* nuclei falls below 10 %. This could not be verified experimentally because attempts to synthesize heterokaryons containing less than 6 % *erg-1+* nuclei were not successful.

4. DISCUSSION

These results show that the nuclear proportions of *N. crassa* heterokaryons can change considerably during extended linear growth on synthetic agar media. Growth of (*erg, pan + leu*) heterokaryons on nystatin-supplemented media caused an increase in proportions of *erg,pan* nuclei and culminated in the selection of homokaryotic *erg, pan* mycelia.

Previous attempts to select homokaryons from heterokaryons of *N. crassa* probably failed because the marker genes carried by the component nuclei were highly recessive and selection pressures were inappropriate. In this investigation, the mutant *erg-1* gene carried by *erg, pan* nuclei in heterokaryon B (*erg, pan + leu*) acts as a recessive gene on non-selective media but the normal *erg-1+* allele carried by *leu* nuclei acts as a semi-dominant deleterious gene on nystatin-supplemented media. Nystatin severely limits growth of hyphae whose nuclei carry the *erg-1+* gene and it favours growth of nystatin-resistant hyphae whose nuclei carry the mutant *erg-1* gene. Despite the deleterious effects of the *erg-1+* gene, however, several weeks growth on nystatin-supplemented media were needed to eliminate *erg-1+* nuclei from heterokaryon B; this result indicates that heterokaryosis in *N. crassa* is an efficient mechanism for maintaining genetic diversity and phenotypic plasticity.

The fluctuating nuclear proportions during growth of heterokaryon B on MM and nutritionally supplemented MM were in sharp contrast to the stable nuclear proportions of heterokaryon A on the same media. Fluctuations might have been due to the mutant *erg-1* gene carried by the *erg, pan* component of heterokaryon B. As the nuclear components of heterokaryons A and B were not isogenic, however, it is possible that differences between the two heterokaryons in stability of their nuclear proportions were due to differences at other unidentified gene loci. Similar fluctuations in nuclear proportions during prolonged growth of *N. crassa* heterokaryons have been described by Grindle & Pittenger (1968).

'Nuclear selection' (Rees & Jinks, 1952) and 'hyphal selection' (Beadle & Coonradt, 1944) were suggested to explain the changes in growth rate and nuclear proportions of heterokaryotic fungi. Nuclear selection occurs when changes in nuclear proportions result from unequal division rates of genetically different nuclei within hyphal tips. Hyphal selection occurs when some hyphae at the growth front of a heterokaryon grow better than their neighbours, and the nuclear proportions of the selected hyphae determine the nuclear proportions of the growing mycelium.

Nuclear selection, due to asynchronous division of *erg, pan* and *leu* nuclei, can account for the fluctuations in nuclear proportions during growth of heterokaryon B (Fig. 3). However, cytological studies have shown that the nuclei in hyphal tips of homokaryotic and heterokaryotic fungi undergo near-synchronous division (Rees & Jinks, 1952; Flentje, Stretton & Hawn, 1963; Clutterbuck, 1970; Trinci, 1979). Fluctuations in nuclear proportions might reflect variations among regions of mycelia (Table 2) resulting from random nuclear assortment during hyphal branching and protoplasmic streaming. It has been suggested that regions of mycelia, rather than individual hyphae, might differ in the timing of nuclear divisions (Davis, 1966; Trinci, 1979). Thus, it is feasible that fluctuations in nuclear proportions are due to chance variations in genetic constitution of the population of nuclei in mycelia and are analogous to random drift of gene frequencies in small, isolated populations of diploid organisms (Wright, 1931).

The mechanism of hyphal selection requires variations in nuclear proportions and growth rate among apical hyphae, and it implies autonomy of individual hyphal tips so that the growth rate of an apical hypha is determined by its own nuclear proportions. Ryan, Beadle & Tatum (1943) and Pittenger & Atwood (1956)

showed that the growth rates of *N. crassa* colonies depended on a zone of mycelium extending approximately 1 cm behind the growth front. Protoplasmic streaming presumably ensures transport of metabolites to hyphal tips so that their functional autonomy, and any growth advantage they might have, is transient. Davis (1966) proposed that growth of hyphal tips is less dependent on the proximal zone of mycelium when rates of growth and protoplasmic streaming are reduced; thus, the autonomy of hyphal tips should be inversely proportional to the rate of hyphal extension. Grindle & Pittenger (1968) observed large increases in growth rate associated with large increases in proportions of *cot*$^+$ nuclei when growth of *N. crassa* heterokaryons containing temperature-sensitive (*cot*$^-$) and normal (*cot*$^+$) nuclei fell below 10 mm/24 h.

Hyphal selection is the more plausible explanation for increases in proportions of *erg, pan* nuclei and selection of homokaryotic *erg, pan* mycelia from heterokaryon B on media containing nystatin (Fig. 3). We propose that chance variations in nuclear proportions generated some apical hyphae which, compared with the parent mycelium, had higher proportions of *erg, pan* nuclei and a growth advantage on nystatin-supplemented media. Continued growth in the presence of nystatin maintained selection pressure in favour of hyphae with increasing proportions of *erg, pan* nuclei and increasing resistance to nystatin, and permitted selection of *erg, pan* homokaryons. Hyphal selection occurred when the growth rate of heterokaryons had decreased from approximately 100 to 45 mm/24 h (Fig. 3) and the growth front was very irregular.

The relationship between nuclear proportions and sterol composition was determined by analysing mycelia grown in liquid media, as it was not feasible to analyse the heterokaryons directly for changes in sterol composition during growth along tubes of agar media. Results of this analysis (Fig. 4) suggested that selection of hyphae with increasing proportions of *erg, pan* nuclei represented selection of hyphae with increasing proportions of fecosterol and lichesterol and/or decreasing proportions of ergosterol. Hyphae may become more resistant to nystatin as more fecosterol and lichesterol are incorporated into cell membranes in place of ergosterol. Resistance to nystatin presumably increases rapidly when the proportion of *erg, pan* nuclei exceeds 90 % and there is a sharp decrease in ergosterol for incorporation into membranes.

Mycelia of *erg-1*$^+$ strains accumulate ergosterol and episterol. Mycelia of mutant *erg-1* strains accumulate fecosterol and lichesterol because their defective $\Delta^8 \rightarrow \Delta^7$ isomerase is unable to catalyse the conversion of fecosterol to episterol and some of the fecosterol is converted to lichesterol by enzymes that normally interact with Δ^7 substrates (Morris, Safe & Subden, 1974; Grindle & Farrow, 1978). Heterokaryon B, which contained both normal *erg-1*$^+$ and mutant *erg-1* nuclei, synthesized appreciable amounts of normal (ergosterol and episterol) and mutant (fecosterol and lichesterol) sterols when they contained 6–85 % *erg-1*$^+$ nuclei (Fig. 4). This result suggests that mycelia with only 6 % *erg-1*$^+$ nuclei can produce sufficient $\Delta^8 \rightarrow \Delta^7$ isomerase to convert about half of the available pool of fecosterol to episterol. The episterol and remaining fecosterol are converted to ergosterol and lichesterol, respectively, by normal hydrogenase and dehydrogenase enzymes produced by both *erg-1*$^+$ and *erg-1* nuclei.

There have been comprehensive studies of the genetic control and regulation of

some biosynthetic processes in fungi (for example, see Kacser & Burns, 1973; Barthelmess, Curtis & Kacser, 1974). We are unable to interpret our data in terms of mechanisms regulating ergosterol biosynthesis because complex multiple pathways are involved and there is insufficient information on the enzymes controlling the steps in the pathways (Parks, 1978; Pierce *et al.* 1979).

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