PHYSIOLOGICAL PROPERTIES OF MUTAGEN-INDUCED VARIANTS OF *CANDIDA ALBICANS* RESISTANT TO POLYENE ANTIBIOTICS

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It is generally believed that strains of *Candida* resistant to the polyene antibiotics have not emerged in the 15 yr during which these compounds have been used clinically. However, a recent report (Hejzlar and Výmola, 1970) states that 31 per cent. of *C. albicans* strains isolated clinically failed to be inhibited by 56 units per ml of nystatin. Bodenhoff (1968) also reported a possible development of resistance *in vivo*. Up to now, studies on resistance to polyenes in the *Candida* species have been confined either to obtaining multi-step mutants by means of serial subculture in the presence of increasing concentrations of antibiotic or by the gradient plate technique (see, e.g., Stout and Pagano, 1956; Littman, Pisano and Lancaster, 1958; Lones and Peacock, 1959; Sorenson, McNall and Sternberg, 1959; Hebeka and Solotorovsky, 1962, 1965; Boudru, 1969; Michalska-Trenkner, 1970; Athar and Winner, 1971). It should be noted that not all attempts to make multi-step mutants were successful; for example, Donovan *et al.* (1955) and Littman *et al.* failed to obtain resistant strains of *Candida albicans*. One-step mutation does not seem to have been reported in the *Candida* species. I therefore thought it of interest to investigate the use of mutagenic agents. This paper reports the properties of four one-step mutants obtained by the action of the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (NNG) on a sensitive *C. albicans* strain; a comparison is also made with the properties of a multi-step mutant derived from the same parent strain.

MATERIALS AND METHODS

Candida albicans strains. No. 799-S, the wild-type sensitive parent strain, was isolated from a patient in Fulham Hospital in 1968. No. 799-R, a multi-step, polyene-resistant variant, was derived from the parent by 60 serial subcultures in the presence of nystatin (Athar and Winner). Nos. 799-XL, -XS, -YL and -YS are one-step polyene-resistant mutants obtained from the parent by treatment with NNG. No. 799-XL/rev is a spontaneous back-mutant, which is sensitive to polyenes.

Media. Penassay Agar (Difco) with 0.5 per cent. glucose was used for the population-structure experiments, disk sensitivity testing, and for measuring the minimum inhibitory concentration (MIC) of polyenes, clotrimazole and pyrrolnitrin. Yeast Morphology Agar (Difco) was used for determinations of the MIC of 5-fluorocytosine. A defined medium was used for studies on mycelium formation; it is a modified version of that described by

Received 29 Dec. 1971; accepted 31 Jan. 1972.

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Lebeault *et al.* (1969), and comprises (per l): NH_4Cl 2.5 g; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 8 g; Na_2HPO_4 7.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g; NaCl 0.1 g; powdered yeast extract (Oxoid) 0.1 g; glucose 2 g. It was sterilised by filtration through a membrane of mean pore size 0.45 μm .

Bismuth-glycine-glucose medium was made according to Nickerson (1953). For all other experiments, a Sabouraud-type medium was used, composed of mycological peptone 10 g, glucose 40 g, and agar (if required) 20 g per litre.

Identification methods. Formation of germ tubes, fermentation reactions and growth on corn-meal and tetrazolium media were examined by standard techniques (see Denny and Partridge, 1968). Assimilation of carbon compounds was investigated by the method of Van der Walt (1970). Various carbon compounds were added, to give approximately 30 mm substrate, to 20-ml amounts of Yeast Nitrogen Base (Difco) containing molten 1.5 per cent. agar; but soluble starch was used at a concentration of 1 per cent. in the same medium. Plates were poured, and water-washed suspensions of the strains to be tested were streaked on the surface of the medium. Each plate was read after 2 and 5 days' incubation at 37°C, growth being compared with that on the control plate, which contained no added source of carbon. If there was growth by the 5th, but not the 2nd, day, it was classified as "late".

Antifungal agents. Amphotericin B (84.4 per cent. pure) and nystatin (4280 units per mg) were supplied by E. R. Squibb & Son, Twickenham, Middlesex. Sensitivity disks containing nystatin (200 μg per disk) and amphotericin (20 μg per disk: "Fungilin") were obtained from Mast Laboratories, Ltd, Liverpool. Filipin (crystalline complex, 96 per cent. pure) was supplied by The Upjohn Co., Kalamazoo, Mich., USA. Pyrrolnitrin was given by Eli Lilly & Co., Indianapolis, USA. Clotrimazole was supplied by FBA Pharmaceuticals, Haywards Heath, Sussex. 5-Fluorocytosine was given by Roche Products Ltd, Welwyn Garden City.

The polyene antibiotics were dissolved in dimethyl sulphoxide, nystatin at 10,000 units per ml, amphotericin B and filipin at 2 mg per ml, and kept frozen at 4°C. Pyrrolnitrin and clotrimazole were dissolved in ethanol at 5 mg per ml; fresh solutions of these compounds were prepared each time they were used.

Minimum inhibitory concentrations. These were measured by a standard plate dilution test, the antifungal agents being incorporated in agar in appropriate concentrations. Yeast strains were grown for 24 hr statically at 37°C in 10-ml volumes of broth and homogenised with a vortex mixer; one loopful of each culture was streaked on to every plate. Plates were read after 40 hours' incubation at 37°C. Up to ten strains could be tested in a single experiment. Control experiments showed that dimethyl sulphoxide and ethanol up to concentrations of 4 per cent. and 1 per cent. respectively had very little effect on the growth of the strains used.

Sensitivity testing with disks. Plates containing 20 ml of Penassay Agar were flooded with a 1 in 10 dilution in broth of a 24-hr broth culture. The excess was sucked off, the plates were dried at 37°C for 1 hr and disks containing nystatin and amphotericin were placed on their surface. The diameters of the zones of inhibition were measured (average of two measurements at right angles) after 18 hours' incubation at 37°C.

Wet and dry weight determinations. The organisms were deposited from suspension by centrifuging in weighed tubes at 1500g for 15 min. The pellets were washed twice in 10-ml amounts of ice-cold water, thoroughly drained, weighed, dried at 105°C overnight and weighed again.

Growth rates. (i) In liquid culture. 20-ml amounts of broth contained in 250-ml screw-capped bottles were warmed to 37°C and inoculated with 1 ml of a 24-hr broth culture. The bottles were shaken at 125 r.p.m. at 37°C in a Gallenkamp orbital incubator; bottles were removed at intervals and the growth of the culture was assessed both nephelometrically and by the dry weight. Log dry weight was plotted against time, and the mean doubling time of the culture during the exponential phase of growth was calculated.

(ii) On solid medium. Cultures were grown for 24 hr in broth and diluted in water so that 1 ml contained about 200 colony-forming units (the dilution factor was about 2×10^5). This dilution was spread—0.1 ml per plate—on four agar plates, which were incubated at 25°, 30°, 37° and 40°C. The plates were inspected at intervals under a plate microscope at a

magnification of $\times 7.5$. As soon as colonies became visible (i.e., had a diameter 0.2 mm or greater) they were numbered on the back of the petri dish, and their diameters measured by means of an eyepiece graticule in the microscope, which was calibrated by means of a Perspex millimetre scale. Colonies were measured thereafter every 24 hr. Means were taken and growth curves constructed for each strain at each temperature.

Cell yields at different temperatures. Sixteen tubes containing 9.5 ml of broth were each seeded with 0.5 ml of a 24-hr broth culture. A set of four of these tubes was incubated at each of the temperatures 25°, 30°, 37° and 41°C; one bottle from each set was removed after 18, 24, 42 and 66 hours' incubation, and the turbidity determined. Extinctions were converted to mg dry weight per ml by the use of a factor determined for each strain in a separate series of experiments (see below).

Population structures. Serial decimal dilutions to 10^{-6} were first made of 24-hr broth cultures; 0.1-ml amounts of the undiluted culture and of each of the dilutions were then spread on the surface of agar plates containing various concentrations of nystatin (from 400 units per ml downwards). The plates were incubated at 37°C for 40 hr and the colonies counted. Control counts (10^{-5} and 10^{-6} dilutions) were made in duplicate on plain agar. From the results, the proportion of organisms in the original culture capable of growing in the presence of each concentration of nystatin was calculated.

Correlation of dry weight, wet weight, viable count and turbidity of suspensions. Cultures in 200-ml amount were incubated for 24 hr at 37°C; they were then spun down and the organisms transferred to weighed tubes. These were washed twice with 10 ml of water and resuspended in 5 ml of water. From a sample of 0.1 ml a viable count was made and the extinction at 700 nm (E700) measured on a Spectronic 20 colorimeter in a 1-cm tube. The suspensions were again centrifuged, the supernate removed and the tubes drained thoroughly and weighed. The tubes were then dried overnight at 105°C and weighed again. From the results, the mean wet and dry weights of one colony-forming unit of each strain were calculated. Determinations were carried out several times on separate cultures of each isolate.

Agglutination. Broth cultures (20 ml) were incubated at 37°C for 48 hr; the cells were spun down, washed in water and resuspended in 5 ml of 1 per cent. formalin in saline and kept at 4°C overnight. The cells were once again spun down, washed twice in 10 ml of saline, and resuspended in saline at a concentration of 4×10^7 cells per ml, as judged by E700 measurements. Volumes (0.03 ml) of these suspensions were added to serial doubling dilutions (0.2 ml in saline) of an anti-*C. albicans* antiserum (prepared by Dr Ruth Evron by injecting formalinised cells of a *C. albicans* type A strain intravenously into a rabbit) in plastic haemagglutination trays. The trays were incubated at 37°C for 2 hr, kept overnight at 4°C and read for agglutination. The same antiserum was also titrated against these suspensions after absorption with the parent strain S. This was carried out as follows: washed cells from a 20-hr, 37°C culture were heated at 65°C for 5 hr, and the equivalent of 10 mg dry weight (judged by the E700) was added to each 1 ml of antiserum. The mixture was incubated at 37°C for 3 hr. The cells were spun down and the antiserum was absorbed twice more in the same way.

Shape and size of cells. Cultures were incubated at 37°C until they had reached the stationary phase. A loopful of each culture was put on a slide and photomicrographs were taken under phase contrast of the wet preparations with a total magnification of $\times 80$. Enlargements ($\times 6$) were made of the negatives, and measurements were taken by means of a plate microscope (magnification $\times 7.5$) equipped with a calibrated eyepiece graticule. Both the major and minor axes of about 20 separate cells of each strain were measured. The mean dimensions and the mean value of the index of ellipticity (major axis/minor axis) were calculated for each strain, and the mean cell volume was calculated from these, assuming each cell to be an oblate spheroid.

Mutagenesis. (a) With NNG. Prewarmed broth, 100 ml in a 500 ml conical flask, was inoculated with 2×10 ml broth cultures of *C. albicans* strain 799-S which had been incubated statically at 37°C overnight. The flask was shaken in the orbital incubator at 125 rev. per min. at 37°C and growth was followed nephelometrically. After $4\frac{1}{2}$ hr, when the viable count

had risen to 2.1×10^7 cells per ml, the culture was spun down, washed in 60 ml tris-HCl buffer (0.1M with respect to tris, pH 8.2) and resuspended in 80 ml of the same buffer. A sufficient amount of the suspension (prewarmed to 37°C) was then added to a weighed amount of NNG to obtain 100 µg per ml of the latter, and the mixture was incubated at 37°C in a waterbath for 1 hr. Treated suspension (1 ml) and an equal volume of control suspension (incubated at 37°C in the presence of tris buffer alone) were inoculated into separate 100-ml amounts of broth and incubated statically at 37°C for 40 hr to allow phenotypic expression to take place. Viable counts were made of each culture, and 0.1-ml amounts of undiluted culture were also plated in duplicate on agar containing 100 units per ml of nystatin. Colonies were counted after 40 hours' incubation at 37°C.

(b) With ultraviolet light. Cells were collected by centrifugation from 200 ml of an overnight broth culture that had been incubated statically at 37°C, washed with 20 ml water and resuspended in 25 ml water. Two 10-ml amounts of the suspension (which had a viable count of 2.3×10^7 cells per ml) were pipetted into separate glass petri dishes (9 cm diameter) and irradiated for 5 and 7.5 min. respectively. The source of ultraviolet light was a Hanovia Chromatolite (45 watt) lamp, and the plates were 40 cm from it during irradiation. Portions (1 ml) of each irradiated sample and of the untreated suspension were seeded into separate 200-ml amounts of broth, which were incubated at 37°C for 24 hr. Viable counts were made, and 0.1-ml samples plated in duplicate on agar containing 100 units per ml of nystatin. Colonies were counted after 40 hours' incubation at 37°C.

Stability of strains. This was investigated in two ways. All the isolates were subcultured three times weekly on slopes which were maintained at 37°C. Eventually, each isolate had been subcultured 46 times. The parent and two of the mutants, YL and YS, were also passaged through the chorioallantoic membrane of chick embryos by Dr Betty Partridge; 48-hr broth cultures of each strain were washed in water and diluted in sterile saline to contain about 5×10^5 organisms per ml; 0.1-ml volumes were then inoculated into suitably prepared 9-day chick embryos (Partridge, Athar and Winner, 1971), which were further incubated for 10 days at 37°C. The chorioallantoic membranes were removed under aseptic conditions and spread over the surface of agar plates. After overnight incubation at 37°C the organisms were transferred to slopes. The subcultured and passaged strains were tested in parallel with the original strains for resistance, mycelium formation in the defined medium and for growth on Nickerson's medium.

RESULTS

Mutation to polyene resistance. Treatment of *C. albicans* strain 799-S with NNG (100 µg per ml) at pH 8.2 for 1 hr at 37°C resulted in the killing of 97.5 per cent. of the original inoculum. After NNG treatment, four mutants (see below) were isolated that were capable of growing in the presence of 100 units per ml of nystatin. The frequency of NNG-induced mutation in this experiment was 1 per 3.3×10^5 cells. No nystatin-resistant colonies were isolated from cultures that had not been treated with NNG. Treatment with ultraviolet light for 5 and 7.5 min. caused the death of 95.1 per cent. and 99.3 per cent. of the cells respectively, but no mutants were isolated from the progeny of the survivors.

Isolation of mutants. Two mutant colonies were isolated from NNG-treated cultures of strain 799-S. When these colonies (called X and Y) were plated out on Sabouraud agar each gave a mixture of large and small colonies, which then bred true on subsequent subculture. On the other hand, when the largest and smallest colonies from a plate inoculated with either strain 799-S or the multi-step mutant 799-R were subcultured, mixtures of large and small colonies were invariably obtained. It was therefore apparent that the original colonies

X and Y were in fact mixed colonies, and that four distinct mutants had been isolated. These were named XL, XS, YL and YS (the L and S suffixes standing for "large" and "small"). Large colonies measured about 1 mm diameter after 18 hours' incubation, small colonies about 0.3 mm. After 3 mth, and several subcultures, mutants XS and YS gave colonies of the "large" type on plating out on Sabouraud agar, and this was still the situation 9 mth after the isolation of the mutants. However, by that time sufficient of the properties of the four mutants had been determined for it to be established that they were different. Others have also observed that small-colony mutants of *C. albicans* induced with NNG revert to large-colony forms on subculture (Savage and Balish, 1971), and the phenomenon reported above is probably a further instance of this type of short-term phenotypic instability.

About 6 mth after the mutants had been isolated, it was noticed that a fresh subculture of strain 799-XL appeared to have lost its resistance to polyene antibiotics. This spontaneously revertant isolate was called XL/rev. The culture from which XL/rev arose was examined, and was found still to be resistant.

In view of this spontaneous back-mutation, and the results obtained indicating that resistance may be lost on serial subculture, routine subculture of the resistant strains was limited to one subculture every 6 mth, and all previous slopes were retained and stored at 4°C.

Characterisation of mutants. The parent and the resistant mutants were characterised as *C. albicans* by the usual laboratory tests. Germ-tube formation in serum was less pronounced in the mutant strains. Fewer of the individual cells formed germ tubes and they tended to be shorter than those produced by the parent strain. On corn-meal agar, strains S and R formed large amounts of mycelium, with many blastospores and well-defined chlamydospores, within 2 to 3 days at 30°C. Mycelial growth was clearly visible to the naked eye after 4 to 5 days. The other strains formed much less mycelium, with correspondingly fewer blastospores; chlamydospores tended to be borne on much shorter lengths of mycelium in the mutants than in the parent. Similar results were obtained when cultures were incubated at 25°C. All grew in a similar fashion on tetrazolium agar. They all had similar fermentation patterns, acid and gas being produced from glucose and maltose, and acid being produced late—on the 5th day—from sucrose. Gas production was less and delayed in the resistant mutant strains. The following carbon compounds were assimilated by the parent strain 799-S: trehalose, glycerol, adonitol, melezitose (weakly), succinate, maltose, xylose, mannitol, sorbitol (late), sucrose, arabinose (weakly), galactose, starch (weakly and late), glucose, sorbose and citrate. The one-step resistant mutants tended to show less ability to assimilate succinate and citrate; when these were the sole sources of carbon growth occurred generally both late and to a smaller extent than with the parent strain under the same conditions. The variants were also sent for identification to the Mycological Reference Laboratory, London School of Hygiene, where they were all reported to be *C. albicans*, by virtue of "fermentation, assimilation and growth on corn-meal agar". (I am very grateful to the late Dr Ian Murray for this report.) On

Nickerson's medium incubated at 37°C, strains S and R had grown well by 40 hr, and reached their darkest colour after about 64 hr. The one-step resistant mutants grew equally well, but were only a light brown colour compared with the deep chocolate of strains S and R and of naturally occurring *C. albicans* strains. Strain XL/rev grew as well as, and formed as dark colour, as the parent. All the mutants, like the parent, grew equally well under aerobic and anaerobic conditions on Sabouraud's agar.

Nature of growth in defined medium. It was discovered by chance that strain 799-S grew largely in the mycelial phase when incubated statically in the defined medium at 37°C. As the appearance of the NNG-induced resistant mutants on corn-meal agar suggested that they might be less able to grow in the mycelial form than the parent strain, they were cultured in the defined medium. Strains XL and XS resembled strains S and R in growing mainly (but not completely) in the mycelial phase, but mycelium was not observed in the cultures of strains YL and YS. The latter grew almost entirely as yeasts of normal microscopic appearance with a tendency to form clumps of 4–6 cells. A few bizarre, club-like forms, which might perhaps be interpreted as an abortive attempt to produce mycelium, were however observed on occasions. Mycelial growth in this defined medium is not a characteristic displayed by all *C. albicans* strains: only one out of four further strains of *C. albicans* examined showed this type of behaviour. Lebeault *et al.* who used the defined medium to grow *C. tropicalis*, do not state whether growth occurred in the mycelial phase or not, but it may be assumed, as they measured growth in this medium by extinction, that growth was in the yeast phase.

Resistance and cross-resistance of mutant strains. The MICs of three polyene antibiotics and three non-polyene antifungal agents for the parent, the "trained" resistant mutant R, the four NNG-induced mutants and the revertant XL/rev are shown in table I. For the five resistant strains, the mean increases in resistance to nystatin, amphotericin B and filipin were 14.4-, 38- and 4-fold respectively; strain XL/rev had virtually the same sensitivity as the parent. There was no cross-resistance to the non-polyene drugs clotrimazole, 5-fluorocytosine and pyrrolnitrin; indeed, the polyene-resistant mutants seemed to be if anything, more sensitive to clotrimazole than was the parent.

Stability of resistance. The MICs of the three polyene antibiotics for the resistant variants were determined after they had been subcultured 46 times *in vitro* and for strains YL and YS after they had been passaged once through chick chorio-allantoic membrane (table II). There was substantial loss of resistance to nystatin and amphotericin B, but little loss of filipin resistance after serial subculture. *In vivo*, passage did not cause any significant changes in resistance.

One-step mutants that had been passaged *in vivo* and *in vitro* regained the ability of the parent strain to produce a dark colour on Nickerson's medium, but strains YL and YS were still unable, after animal passage, to produce mycelium in the defined medium.

Disk sensitivity testing. With the sensitive parent strain, the usual size of the zone of inhibition was 17–20 mm around the nystatin disk and 12–14 mm

around the amphotericin B disk. Despite the standardised conditions of testing the zone sizes varied from experiment to experiment. To compensate for this,

TABLE I
Minimum inhibitory concentrations of six antifungal agents for Candida albicans strain 799-S and its derivatives

Designation of organism	Minimum inhibitory concentration of					
	nystatin (units per ml)	amphotericin B (μ g per ml)	filipin (μ g per ml)	clotrimazole (μ g per ml)	5-fluorocytosine (μ g per ml)	pyrrol-nitrin (μ g per ml)
799-S	25	1	3	5	0.2	30
799-R	400	40	20	0.25	0.2	30
799-XL	400	30	10	2	0.1	30
799-XS	400	40	10	2	0.2	20
799-YL	300	40	10	0.5	0.2	10
799-YS	300	40	10	0.5	0.2	20
799-XL/rev	35	2.5	5	...	...	...

the results of each experiment were expressed as the ratio of zone diameter observed for the test isolate to that observed with the parent (table III).

The zones around the resistant variants were considerably reduced in size but strain XL/rev showed zone sizes close to those of the parent.

TABLE II
Stability of resistance in variants of Candida albicans strain 799-S

Organism	Previous treatment	Minimum inhibitory concentration of		
		nystatin (units per ml)	amphotericin B (μ g per ml)	filipin (μ g per ml)
R XL XS YL YS	} subcultured <i>in vitro</i> 46 times }	50	3	10
		25	3	5
		50	3	10
		35	3	10
		10	20	10
YL YS	} passed through chorioallantoic membrane }	200	30	10
		200	30	10

The areas of the zones of inhibition around the resistant strains were in every case less than half of the areas of the corresponding zones around the parent strain.

In a previous unpublished pilot survey of 34 *Candida* strains (20 *C. albicans*,

14 *C. tropicalis*) I observed that zone diameters of 17 to 20 mm around a nystatin disk consistently occurred with strains sensitive to 25 or 35 units per ml of nystatin. The zone diameters around amphotericin B disks were between 12 and 14 mm, with MICs from 0.5 to 1.5 μ g per ml. The scarcity of either highly resistant or highly sensitive strains makes it impossible at this stage to establish whether there is a mathematically significant correlation between zone diameter and MIC for these antibiotics.

Population structures. The amount of heterogeneity in nystatin resistance of strain 799-S and the six variants derived from it was measured by determining the proportion of individual cells of each strain able to grow in the presence of various concentrations of antibiotic. For easier interpretation of the results,

TABLE III
Disk sensitivity testing of Candida albicans strain 799-S and variants of it

Organism	Relative diameter* of zone around disk containing	
	nystatin	amphotericin B
S	1	1
R	0.63	0.65
XL	0.68	0.63
XS	0.7	0.67
YL	0.62	0.68
YS	0.63	0.69
XL/rev	0.92	0.9

* Relative to strain 799-S in the same experiment. Means of three experiments.

a graph was constructed for each variant of the log percentage of survivors against the antibiotic concentration, and from these the concentrations of antibiotic necessary to inhibit 50 per cent., 90 per cent., 95 per cent., 99 per cent. and 99.99 per cent., respectively, of the cells of each variant were read off. These figures (IC₅₀, IC₉₀, IC₉₅, IC₉₉ and IC_{99.99}) are presented in table IV. A numerical measure of the degree of heterogeneity for each isolate is given by the ratios of the various IC values to IC₅₀; for a completely homogeneous strain this ratio would be unity. It can be seen that strain R is heterogeneous at higher levels of resistance, and strain XS at intermediate levels, while the remainder are as homogeneous as the parent; strain XL/rev resembles the parent strain in sensitivity.

Size, shape and weight of cells of C. albicans strain 799-S and its derivatives. The dimensions of 18–24 individual cells of each variant were measured from photomicrographs, as described in the *Methods*. The results are given in table V. Cells from the parent strain were not significantly oval in profile (i.e., the mean major axis was not significantly longer than the mean minor axis, $P > 0.1$), while cells of the five resistant variants were significantly oval. Cells from the trained resistant variant, R, were significantly smaller than those of the parent, and cells from the four one-step mutants were significantly larger

than those of the parent ($P < 0.001$). They were between 1.8 and 3.5 times greater in volume than were the cells of the parent.

The dry and wet weights of cells are also given in table V. Cells of the mutants XS, YL and YS were of significantly greater mass than those of the parent strain ($P < 0.05$), in both the wet and dry states. Cells of strain YS were heavier than cells from any other strain ($P < 0.001$); cells of strain XL/rev were significantly lighter ($P < 0.05$) than cells of strain XL. For all the isolates, the ratio of wet weight to dry weight was very close to 5:1. Taken by themselves, the figures for wet and dry weights could be interpreted simply as meaning that some of the mutant cells formed clumps, so that a single colony-forming

TABLE IV

Concentrations of nystatin required to inhibit 50 per cent., 90 per cent., 95 per cent., 99 per cent. and 99.9 per cent. of cells in Candida albicans strain 799-S and in resistant variants of it

Organism	Value (in units per ml) of				
	IC50	IC90	IC95	IC99	IC99.99
S	11	15 (1.36)*	17 (1.55)	21 (1.9)	33 (3)
R	60	78 (1.3)	86 (1.43)	116 (1.94)	320 (5.35)
XL	130	194 (1.49)	210 (1.61)	244 (1.88)	300 to 400
XS	88	250 (2.84)	278 (3.16)	300 to 400	300 to 400
YL	64	96 (1.5)	110 (1.72)	140 (2.18)	200 to 300
YS	176	242 (1.37)	262 (1.49)	300 to 400	300 to 400
XL/rev	11.0	12 (1.1)	12.5 (1.14)	16.5 (1.5)	21 (1.9)

* In parentheses, proportional increase over respective IC50 value.

unit represents more than one viable cell. If this were true, the density of each colony-forming unit from strains XS, YL and YS would be from 1.8 to 3.5 times greater than that of a cell of the parent strain, but this was not the case (table V). These experiments show that cells of these strains are both larger and heavier than those of the parent strain.

Growth rates. (a) In liquid medium. The mean doubling times in minutes (estimated from the dry weight) in shaken cultures at 37°C were: for strain S: 94; strain R: 90; strain XL: 126; strain XS: 127; strain YL: 130; strain YS: 118 and strain XL/rev: 90. Under these conditions, specific growth rates ranged from 0.461 to 0.32 hr⁻¹. The rate of growth of the parent and of the trained variant was higher than that of the one-step resistant mutants by a factor of about 1.35; it is noteworthy that the sensitive revertant from strain XL had a higher rate of growth than its resistant parent.

(b) On solid medium. Plots were made of mean colony diameters against time for the parent, and for the trained and the one-step resistant mutants. The usual pattern of such plots was a lag phase followed by a period in which the increase in mean colonial diameter was linear with time; this linear phase was usually followed by a cessation in growth at the higher incubation temperatures. At 40°C the linear phase usually lasted only 2 or 3 days, at 37°C

for 3 or 4 days, at 30°C until the 4th to the 7th day, and at 25°C it continued to the 9th day of observation, at which point the experiment was discontinued. At this stage, colony diameters were at least as great (from 4·7 to 9 mm, depending on the isolate) as those observed on plates that had been incubated at the higher temperatures.

The duration of the lag phase was measured on each plot by producing back the linear part of the growth curve to zero colony diameter; these figures, together with the rates of radial growth observed during the linear phase, are given in table VI. There is a tendency for the lag phase to decrease and the growth rate to increase as the incubation temperature rises from 25°C to 37°C,

TABLE VI
Growth of colonies of Candida albicans strain 799-S and variants of it on Sabouraud agar at different temperatures

Organism	Growth at an incubation temperature of							
	25°C		30°C		37°C		40°C	
	Lag phase*	Growth rate	Lag phase*	Growth rate	Lag phase*	Growth rate	Lag phase*	Growth rate
S	0·7	0·98	0	1·09	0	1·33	0·2	1·2
R	0·9	1·03	0·5	1·44	0·3	1·13	0·6	1·55
XL	1·2	0·61	1·0	0·88	0·4	0·94	0·5	0·9
XS	1·6	0·58	1·0	0·81	0·8	1·05	0·9	0·8
YL	0·9	1·2	0·6	0·98	0·5	1·08	0·7	0·9
YS	1·9	0·8	1·1	0·92	0·7	1·25	0	0·87

* Lag phase in days;
† Growth rate in mm per day; } for methods of determination see text.

but a further rise to 40°C generally reverses this trend. Palumbo *et al.* (1971) have suggested that, for growth on the surface of solid media, linear increase of colonial diameter with time is equivalent to exponential growth in liquid medium.

(c) Cell yields. In static culture (10 ml broth in a 25-ml Universal Container), maximum cell yields occurred after 42 or 66 hr of growth at either 25°C or 30°C. Yields were lower at 37°C and lower still at 41°C. Differences in yields between the isolates were virtually non-existent at 25°C, but became more marked as the temperature increased. Strains S and XL/rev usually grew better than the other isolates.

The yields at 37°C after 24 hr (between 0·5 and 0·9 mg dry weight per ml) under the conditions described above were some three times greater than those obtained after 24 hours' incubation at 37°C when 200 ml of broth was contained in a 250-ml bottle. Still higher yields were obtained when 20 ml amounts of broth were shaken for 24 hr in the orbital incubator in 250 ml bottles; under these conditions, as much as 5 mg dry weight per ml was obtained.

Agglutination. Suspensions of strain 799-S and of the five resistant variants were titrated against a specific *C. albicans* antiserum before and after it had been absorbed with cells of strain 799-S (table VII). The titres for each isolate were within one two-fold dilution of each other, and absorption of the antiserum lowered the titres by between 16- and 32-fold in each case. Hence, there is no evidence of serological differences between the parent and mutant isolates.

TABLE VII
Titre of agglutination of Candida albicans strain 799-S and of variants of it by antiserum before and after absorption by strain 799-S

Antiserum	Titre of agglutination, by the stated antiserum, of strain					
	S	R	XL	XS	XL	YS
Unabsorbed	512	512	256	256	256	256
Absorbed	16	16	8	16	8	16

DISCUSSION

Alkylating agents, such as ethyl methanesulphate, N-methyl-N'-nitro-N-nitrosoguanidine and N-nitroso-N-methylurethane, have been very widely used as mutagens for many types of micro-organisms, mainly bacteria and haploid yeasts. Nutritional studies have been made on mutants from species such as *Saccharomyces cerevisiae* (Lingens and Oltmanns, 1964), *Schizosaccharomyces pombe* (Megnet, 1965), *Candida pelliculosa* and *C. guilliermondii* var. *membranaefaciens* (Tsukada and Sugimori, 1971); but, according to van Uden and Buckley (1970), the latter two species should be named respectively *Hansuela anomala* var. *anomala* and *Pichia ohmeri*. NNG is mutagenic for blue-green algae and *Euglena gracilis* (Aldrich Technical Bulletin on NNG, 1970) as well as for animal cells (e.g., Chu and Malling, 1968). Recently, Savage and Balish (1971) used alkylating agents to make auxotrophic mutants from the diploid yeast *C. albicans*. However, mutagens have apparently not been used before to induce drug-resistance in yeasts.

Alkylating agents have generally proved to be better mutagens for yeasts than, for instance, nitrous acid, hydroxylamine or ultraviolet radiation (Lingens and Oltmanns; Lindegren *et al.*, 1965; Savage and Balish; Tsukada and Sugimori). In the experiments here described mutagenesis with NNG was carried out at pH 8.2 instead of the more acid conditions usually used, in view of the reports of Lingens and Oltmanns (1964) and Delić, Hopwood and Friend (1970) that alkylating agents are more mutagenic at an alkaline pH.

Previous studies indicate that not all strains of *C. albicans* can be induced to acquire resistance to the polyene antibiotics. Donovan *et al.* (1955) and Littman *et al.* (1958) failed to obtain *C. albicans* strains resistant to nystatin and amphotericin B, and Athar and Winner (1971) succeeded in "training" less than half the strains tested to resistance to these two antibiotics. The parent

strain used in the present experiments had been shown (Athar and Winner) to possess a predisposition to acquire resistance.

The degree of polyene resistance obtained in the present experiments (12- to 16-fold against nystatin, 30- to 40-fold against amphotericin B, and 3- to 7-fold against filipin) resembles that obtained by other workers, all of whom used "training" techniques on *C. albicans*. Stout and Pagano (1956), Lampen, Morgan and Slocum (1957), Hebeka and Solotorovsky (1965), Patel and Johnson (1968), Bodenhoff (1969) and Boudru (1969) reported increases in nystatin resistance of 2- to 6-fold, while Athar and Winner obtained rather greater values (30- to 40-fold). Resistance to amphotericin B appears to reach higher levels. Lones and Peacock (1959) and Hebeka and Solotorovsky reported increases respectively of 29- and 60-fold, while Sorenson, McNall and Sternberg, and Athar and Winner reported increases of more than 1000-fold. The latter authors found only a 5-fold increase in resistance to filipin. Thus, my results with one-step mutants are broadly in line, both qualitatively and quantitatively, with those found in "trained" strains. That resistance to one polyene confers resistance to other polyenes is hardly surprising, in view of the similarities in chemical structure and mode of action of this group of antibiotics. It is interesting, however, that filipin ($C_{35}H_{48}O_{11}$; molecular weight 654.9), a relatively small polyene, has been found (see Lampen, 1969) to bring about much more, far-reaching damage to the membranes of susceptible organisms than do either of the larger polyenes, amphotericin B ($C_{47}H_{72}O_{17}N$; mol. wt 922.1; Mechliniski *et al.*, 1970) and nystatin ($C_{47}H_{75}O_{17}N$; mol. wt 925.1; Borowski *et al.*, 1971); this may account for the fact that resistance to filipin is less well developed than is resistance to the other two polyenes under consideration.

The modes of action of 5-fluorocytosine (interference with pyrimidine metabolism; Jund and Lacroute, 1970) and pyrrolnitrin (blockage of mitochondrial electron transfer; Wong and Airall, 1970) involve intracellular sites, and are unrelated to the mode of action of the polyenes. The mode of action of clotrimazole is not known, but may involve the intracellular formation of carbonium ions (Plempel *et al.*, 1969). Any cross-resistance would presumably be attributable to a decreased permeability on the part of the polyene-resistant cell. The results obtained show that cross-resistance, and hence decreased permeability, does not occur. Hebeka and Solotorovsky (1965) also found that *C. albicans* trained to resistance to candidin or to amphotericin B did not gain resistance to the non-polyene antibiotics euiclin and griseofulvin.

In many respects the one-step mutants seem to be less vigorous than the parent; they grow more slowly, ferment less rapidly, assimilate the tricarboxylic acid cycle intermediates citrate and succinate less well, and reduce bismuth sulphite less well than the parent strain. Loss of resistance, as exemplified by strain XL/rev, results in restoration of certain of these capabilities. There was no difficulty, however, in identifying the mutant isolates as *C. albicans*, as their basic biochemical and serological characteristics had not altered greatly; however, two of the mutants, YL and YS, have lost, to a significant extent, their capacity to produce mycelium. If a *C. albicans* strain lost completely

the ability to form pseudomycelium, classification would present a considerable problem; as van Uden and Buckley (1970) point out, the genera *Torulopsis* and *Candida* are differentiated chiefly by the ability of the latter to form pseudomycelium. Hence, a myceliumless mutant of *C. albicans* could, on the basis of fermentation patterns, be identified as *T. etchellsii*, *T. mogii* or *T. versatilis* (cf. van Uden and Buckley, 1970; van Uden and Vidal-Leiria, 1970). There are four points of difference between the assimilation patterns of 13 substrates by *C. albicans* and *T. etchellsii*, and three between *C. albicans* and *T. versatilis*; however, *C. albicans* differs conclusively from *T. mogii* only in the former's ability to assimilate D-xylose.

Thus, it is conceivable that a myceliumless mutant of *C. albicans* might be misidentified. If inability to form mycelium were linked, either causally or coincidentally, with polyene resistance, such a strain need not necessarily be identified as a polyene-resistant *C. albicans*, and might indeed be passed off merely as a commensal without sensitivity testing. It is not suggested that this explanation accounts for the alleged absence of polyene-resistant *C. albicans* strains in nature, but it might none the less be worth carrying out sensitivity tests on "commensals" isolated from patients, especially those undergoing treatment with polyene antibiotics, to determine whether an undiscovered reservoir of polyene-resistant yeast does in fact exist.

SUMMARY

Mutants resistant to nystatin have been selected from cultures of a strain of *Candida albicans* isolated from a patient after it had been treated with N-methyl-N'-nitro-N-nitrosoguanidine. The resistance of the mutants to nystatin, amphotericin B and filipin was respectively 12- to 16-fold, 30- to 40-fold and 3- to 7-fold, more than that of the parent strain. Sensitivity to three non-polyene compounds, clotrimazole, 5-fluorocytosine and pyrrolnitrin, was not altered. The mutants were identifiable as *C. albicans* by standard laboratory tests; two of them seemed to have a decreased ability to form mycelium. There was some loss of acquired resistance after 46 passages *in vitro*. The mutants had more homogeneous patterns of resistance than did a resistant isolate of the same parent strain which had been serially subcultured in the presence of increasing concentrations of nystatin. Mutant cells were found to be of greater eccentricity, and of greater total volume and mass, than those of the parent strain. In shaken culture at 37°C the mutants grew more slowly than the parent; decreased growth rates and prolonged lag phases were also noted for the mutants when grown on solid media at four temperatures. The resistant isolates were agglutinated by similar dilutions of an antiserum to *C. albicans*, and the agglutinins were removed by absorption of the antiserum with the parent strain. Possible taxonomic differences arising from loss of mycelium-forming ability of *C. albicans* strains are discussed.

It is a pleasure to thank Professor H. I. Winner for his help and encouragement. I am also very grateful for the continual advice and assistance of Dr Betty Partridge, Dr Ruth Evron and Miss Maire Denny, and for the technical help of Miss Pat Thorne. This research was carried out during the tenure of a Squibb Research Fellowship, which I gratefully acknowledge.

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