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ULTRASTRUCTURE, COMPOSITION OF NEUTRAL LIPIDS AND THEIR FATTY ACIDS OF *CANDIDA TROPICALIS* STRAIN D-2 MUTANTS RESISTANT TO THE POLYENE ANTIBIOTIC NYSTATIN

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This report deals with data on the cell ultrastructure of *Candida tropicals* strain D-2 mutants resistant to the polyene antibiotic, nystatin, and with an analysis of the fractional composition of neutral lipids and their fatty acids. The ultrastructural organization of the mutant cells is characterized by thickening of the cell wall and formation of invaginations into the cytoplasm, the appearance of new formations, large vacuoles, and reduction of the system of mitochondrial cristae. Lipids of *nys*^{r} mutants differ from those of the *nys*^{s} variant in having a decreased content of steroids and some fractions of neutral lipids. Certain *nys*^{r} mutants manifest difference in the relative amounts of saturated and unsaturated fatty acids ($C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$).

Introduction

Polyene antibiotics belong to a group of compounds which affect membranes [1,2]. The availability of strains resistant to these antibiotics obtained using mutagenic factors, as well as interest in the treatment of candidoses, dermatomycoses and other fungal infections [3,4] requires study of their mechanisms of action. Such studies might lead towards the prevention of these infections.

At present, there is no doubt that any mutation leading to the development of resistance to polyenes is connected with disturbances in the structure and function of biological membranes [5–9]. Some microorganisms resistant to various antibiotics of this group have been described [10,11]. Biochemical studies have shown that in polyene-resistant mutants, there occur qualitative and quantitative alterations of steroid composition. Most investigators believe that the mechanism of polyene antibiotic action is connected with the presence or absence of steroids in the cell. The fact that mutants of lower fungi, which show no changes in steroid composition, have already been described shows that this hypothesis is premature [12]. It is also known that polyenes (macrocyclic lactones with conjugated double bonds) possess three characteristic features: (1) an affinity for lipids due to the presence of conjugated double-bond systems; (2) a hydrophilic part of the molecule with a series of hydroxyl groups; (3) an aminosugar [13,14]. The presence of such functional groups might suggest that polyene resistance may be due to some other change in membranes and cells in general. It might appear that examination of model membrane systems [15,16] in the study of this problem will be insufficient. At present, experiments using various techniques are necessary to study not only alterations in steroids of cell membranes resistant to polyene antibiotics, but also changes in other classes of compounds differing in nature and function.

The aim of this work was to study the ultrastructural cell organization of *Candida tropicalis* strain D-2 mutants resistant to the polyene antibiotic, nystatin (*nys*^{r} mutants), and also to analyze

the composition of neutral lipids and determine their fatty acid composition.

Materials and Methods

C. tropicalis strain D-2 was used for the analysis [17]. Mutants resistant to nystatin were isolated by treating the cell with chemical mutagens from a group of nitroso compounds: nitromethylguanidine, nitrosomethylurea, nitrosodimethylurea and nitrosoethylurea.

Cells for electron-microscopic examination and also for obtaining lipids and their further gas-chromatographic and thin-layer analyses were isolated from the original and mutant strains by cultivation in full nutrient medium (bear wort Blg 7°) as described by Balling [17], at pH 7.0–7.2 and thermostatically maintained at 30°C.

The method of preparing cells for electron-microscopic examination has been described previously [18–20]. Cells at the stationary growth phase were washed three times in distilled water, suspended in 1.5% fresh $KMnO_4$ solution, kept for 20–24 h in a refrigerator, periodically shaken, and then repeatedly washed in water at room temperature and dehydrated in a series of alcohols of 30, 50 and 70%. They were then kept overnight in a 2% solution of uranyl acetate in 70% alcohol and suspended in a mixture of methacrylates. Sections were stained with lead citrate and analyzed in an electron microscope (made in the U.S.S.R.) at a magnification of 13000 and also in a JEM-7 microscope (made in Japan).

Lipids were analyzed by two methods: by GLC and TLC as well as by a combination of both. A Tsvet-110 gas chromatograph fitted with a flame-ionization detector (sensitivity $2 \cdot 10^{-10}$) was used. We used a glass column (3 ml) with an inside diameter of 3 mm containing a stationary phase of 1% SE-30 on chromosorb. The temperature of the column was programmed for 30 min from 120 to 250°C at 3 K/min. The gas carrier was nitrogen (rate, 2 litre/h).

Calculations from chromatograms of certain fatty acids were made using internal standardization with known standards expressed as relative percentages [21]. Neutral lipids were separated on TLC plates (Merck, F.R.G.) using the following solvent system, petroleum ether/diethyl ether/acetic acid (90:10:1). Lipid concentrates were placed in plates as strips, appropriate solvents were added and the concentrates placed for 15–20 min in chambers with iodine vapour for detecting spots. A qualitative analysis was made by means of R_f values of standard samples, or by washing chromatograms with reagents specifically demonstrating particular lipid classes using standard samples [22–25]. Qualitative determination of particular fractions of neutral lipids was carried out by the method described in Ref. 26. Lipids for the analyses were obtained by extraction, using a chloroform/methanol mixture [27], and concentration under a stream of nitrogen.

Results

Analyzing the cell ultrastructure of the initial antibiotic-sensitive strain, it can be seen that at the stationary phase of growth the cell wall is relatively thicker (about 2500 Å). Glycogen granules are diffusely arranged in the cytoplasm. Nuclei are relative small and mainly of regular shape. Mitochondria are small, with a poorly developed system of cristae ranging in number from five to ten. The cell wall is three layered, the outer electron-dense layer loose and the inner layer compact (Figs. 1 and 2). Peculiar, electron-dense substrate-filled phenoysosomes with a toothed outline were observed in most cells (Fig. 3).

The cell ultrastructure of mutants differs essentially from that of the initial resistant strain. It is characterized by thickening of the cell wall (Fig. 4). In some cells the thickness of the cell wall reaches 6500 Å (Fig. 5). Deposition of components of the cell wall on its inner surface is found to be non-uniform, which results, presumably, in invagination into the cytoplasm (Fig. 6). The cell wall substrate accumulates in the cytoplasm in the form of round neoplasms without an inner cavity (Fig. 7). Another distinctive feature is enormous lysosomes which become rounded in shape and occupy a large space within the cell. Ultrastructural analysis of *nys*^{r} mutant, possessing a higher degree of resistance, reveals thickening of the cell wall and its lamellar structure (up to six layers) (Fig. 8). In cell sections, one can see accumulation of cell wall substrate in the cytoplasm in the form of large neoplasms (Fig. 9). In some cases these are

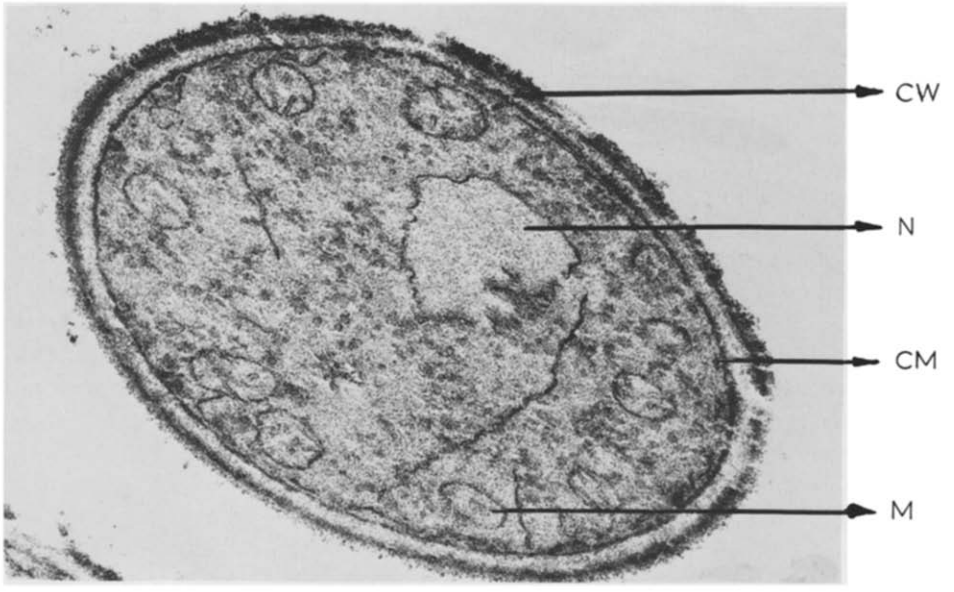

Fig. 1. (Top) Ultra-thin sections of *C. tropicalis* strain D-2 cells sensitive to nystatin. A three-layered cell wall is observed. Magnification ×18000. CW, cell wall; N, nucleus; CM, cytoplasmic membrane; M, mitochondria.

linked with the cell wall (Fig. 10). It should be mentioned that, as a rule, they do not contain cavities, which excludes their relation to possible spore formation. The cells contain vacuoles and have no phagolysosomes. The structure of the mitochondria did not differ from that of control

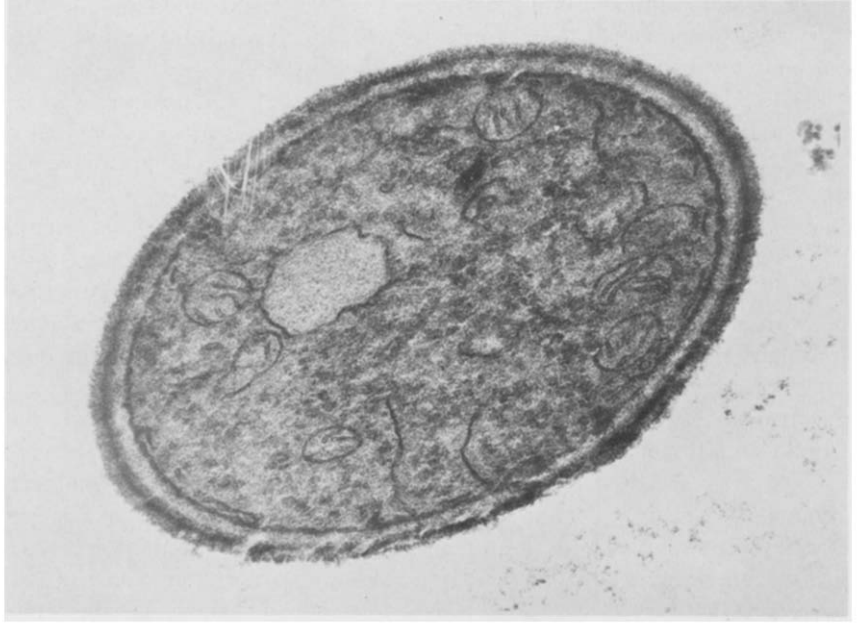

Fig. 2. (Bottom) Ultra-thin sections of *C. tropicalis* strain D-2 cells sensitive to nystatin. A three-layered cell wall is observed. Magnification ×17800.

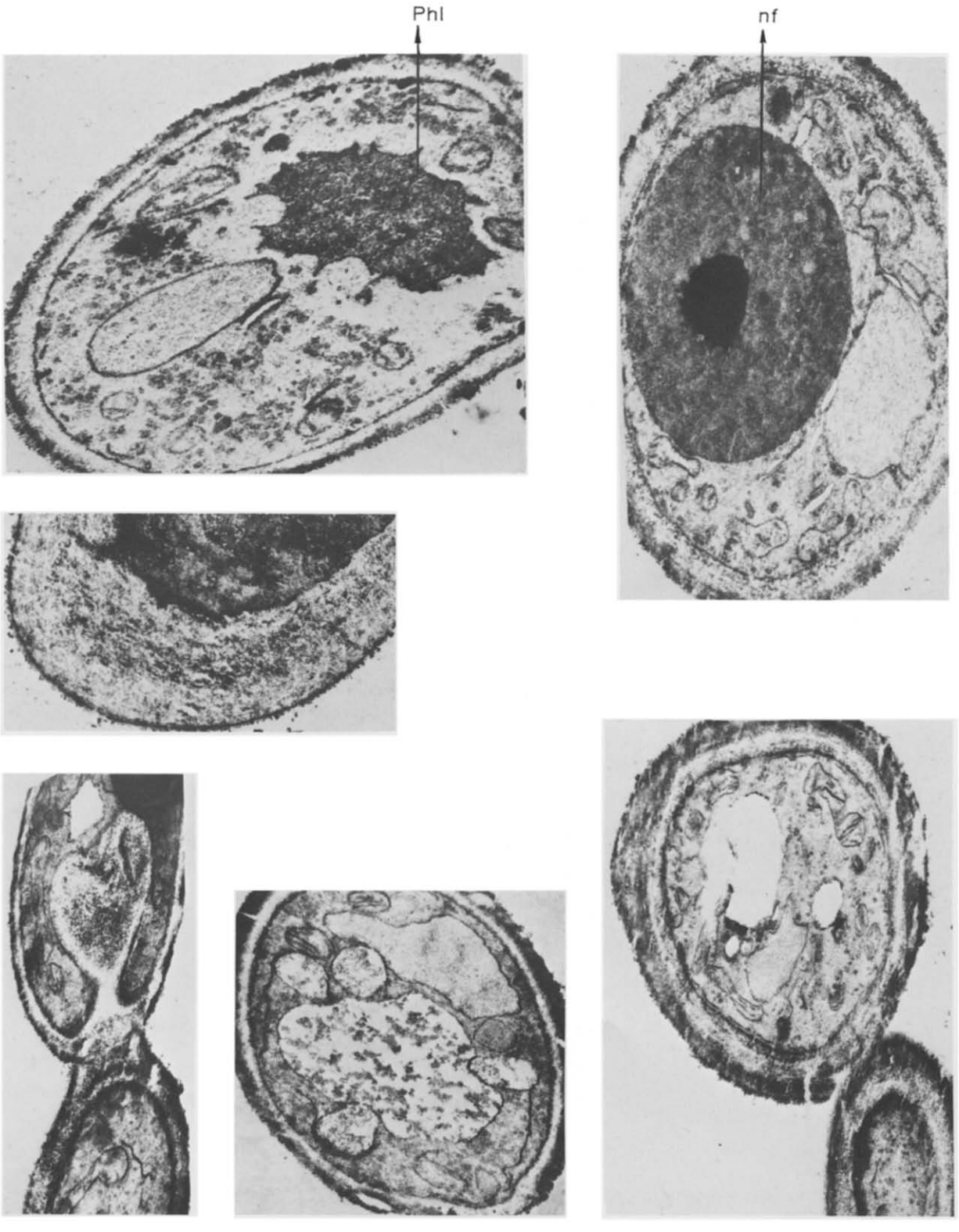
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TABLE I

THE RELATIVE CONTENTS OF DIFFERENT CLASSES OF NEUTRAL LIPIDS IN CELLS OF *CANDIDA TROPICALIS* D-2 VARIANTS SENSITIVE AND RESISTANT TO NYSTATIN

Lipid classes	Phenotypes									
	nys^s	*nys*^{r} 7–10	*nys*^{r} 8–10	*nys*^{r} 10–12	*nys*^{r} 20	*nys*^{r} 30–35	*nys*^{r} 60	*nys*^{r} 50–60	*nys*^{r} 20–25	*nys*^{r} 80
Phospholipids	67.9 ±4.9	66.6 ±3.7	70.7 ±4.0	65.3 ±0.1	67.9 ±4.0	49.1 ±3.0	70.6 ±3.0	68.0 ±4.0	56.3 ±2.0	66.4 ±6.0
1,2-Diacylglycerols	6.55 ±0.92	8.2 ±1.1	6.9 ±1.1	7.9 ±0.1	6.7 ±0.8	9.7 ±1.0	7.0 ±0.6	6.5 ±0.7	10.7 ±1.0	8.4 ±0.7
1,3-Diacylglycerols	2.7 ±0.1	3.3 ±0.6	3.0 ±0.1	3.0 ±0.1	2.8 ±0.1	5.8 ±1.0	6.0 ±0.5	3.9 ±0.4	3.8 ±0.4	4.0 ±0.5
Steroids	9.98 ±0.2	7.8 ±0.9	6.0 ±0.2	4.6 ±0.1	8.1 ±0.3	6.1 ±0.8	3.0 ±0.7	5.4 ±0.6	5.0 ±0.4	7.6 ±0.7
Free fatty acids	4.7 ±0.1	5.07 ±0.4	5.1 ±0.1	5.2 ±0.4	4.7 ±0.4	8.7 ±0.9	5.4 ±0.6	5.8 ±0.9	6.7 ±0.6	4.4 ±0.6
Triacylglycerols	4.2 ±0.6	4.4 ±0.4	4.5 ±0.1	4.3 ±0.2	6.0 ±0.3	11.0 ±1.0	5.2 ±0.1	5.3 ±0.4	9.0 ±0.6	5.0 ±0.3
Ethers of fatty acids	1.5 ±0.1	2.5 ±0.3	1.6 ±0.1	1.7 ±0.1	1.28 ±0.1	6.28 ±0.5	1.5 ±0.2	1.8 ±0.4	2.08 ±0.3	2.6 ±0.3
Hydrocarbons	2.11 ±0.1	1.9 ±0.2	1.8 ±0.3	2.3 ±0.2	2.38 ±0.2	2.66 ±0.3	2.0 ±0.1	2.9 ±0.1	2.38 ±0.1	2.1 ±0.1

cell mitochondria, but the former are very small and have a reduced system of cristae (Fig. 11). The endoplasmic reticulum was prominent. In some cases the amount of mitochondria was greater. Sometimes the invaginations of the cell wall formed partitions which divided cells into compartments (Fig. 12). Cells of other mutants also revealed thickening of the cell wall which had a rather thick outer electron-dense layer, numerous vacuoles and a nucleus of irregular shape (Figs. 13–16). In some instances the cell wall was so thick that preparation for electron microscopy was impossible.

Data showing the composition of neutral lipids of a total lipid fraction of the initial strain and nystatin-resistant mutants are presented in Table I. Lipids of the initial strain are found to contain phospholipids, 1,2- and 1,3-diacylglycerols, steroids, triacylglycerols, free fatty acids, ethers of fatty acids and steroids and also a hydrocarbon fraction. The quantitative distribution of the above-mentioned lipid classes is as follows: phospholipids, 67.8%; 1,2-diacylglycerols, 6.6%; 1,3-diacylglycerols, 2.7%; steroids, 9.9%; free fatty acids, 4.7%; triacylglycerols, 4.2%; ethers of fatty acids

Fig. 3. (Top left) Ultra-thin section of sensitive variants. Phagolysosomes (PhL) with an indented outline are visible. Magnification ×17500.

Fig. 4. (Top right) Sections of mutant *nys*^{r} 20–25. The cell wall is thickened. Magnification ×14500. nf, new formations.

Fig. 5. (Centre) Section of mutant *nys*^{r} 20–25. The cell wall is thickened. Magnification ×11000.

Fig. 6. (Bottom left) Section of mutant *nys*^{r} 20–25. Invagination is observed. Magnification ×14000.

Fig. 7. (Bottom centre) Ultra-thin section of cells of mutant *nys*^{r} 20–25. Large neoplasms are observed. Magnification ×17300.

Fig. 8. (Bottom right) Ultra-thin section of mutant *ny*ys^r 20–25, showing the lamellar nature of the cell wall and its thickness. Magnification ×12500.

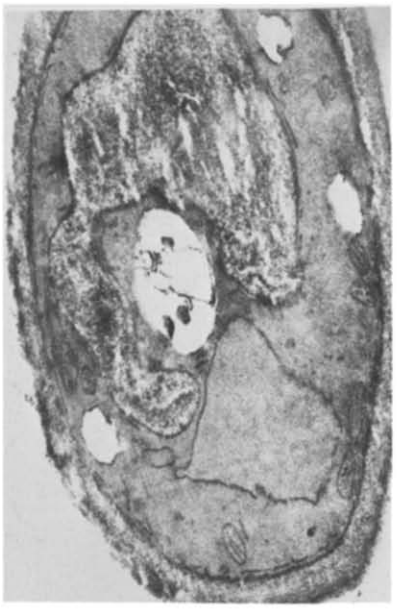


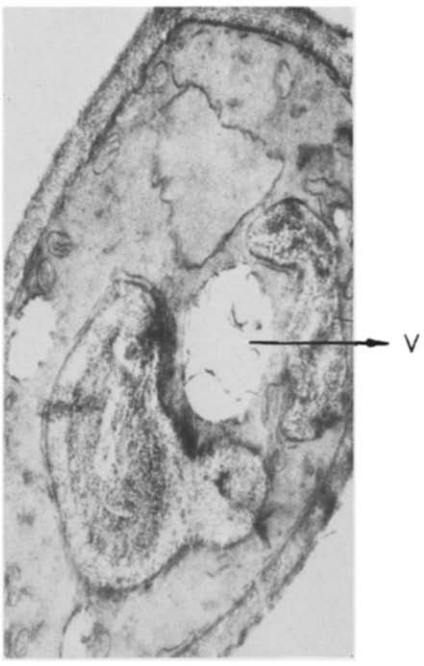
Fig. 9. (Top left) Accumulation of cell wall substrate in cytoplasm. Magnification ×14000.

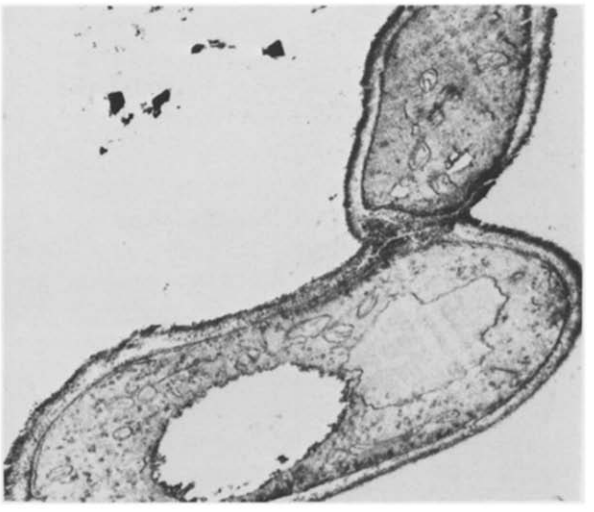
Fig. 10. (Centre) Cell wall substrate linked with substrate in cytoplasm. Magnification ×14000.

Fig. 11. (Top right) Ultra-thin section of mutant *nys*^{r} 20, showing reduction of cristal system. Magnification ×14000.

Fig. 12. (Bottom left) Ultra-thin section of mutant *nys*^{r} 20, showing the formation of partitions and division of the cell wall. Magnification ×15000. IS, inner septum.

Fig. 13. (Bottom right) Mutant cell with three nuclei. Magnification ×14000.





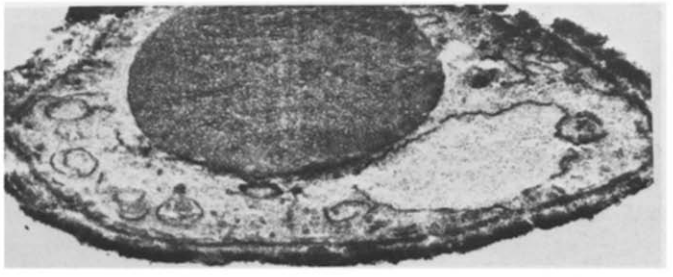


Fig. 14. (Left) Section of mutant *nys*^{r} 50–60, showing the accumulation of substrate in the cytoplasm. Magnification ×14000. V, vacuole.

Fig. 15. (Top right) Ultra-thin section of mutant *nys*^{r} 80. Magnification ×9000.

Fig. 16. (Bottom right) Section of mutant *nys*^{r} 30–35. Magnification ×14000.

and steroids, 1.5%; hydrocarbons, 2.1%. Analysis of the mutants revealed a tendency to a decrease in the relative content of steroids in their lipids. In a total lipid fraction of the control *nys*^{s} variant the decrease amounted to 9.9% and in lipids of mutant *nys*^{r} 7–10 7.8%. The content of steroids in mutant *nys*^{r} 10–12 is half as much. The largest decrease in relative content of steroids was revealed in mutant *nys*^{r} 60. The content of steroids in lipids of this mutant is one-third as much. Changes in the content of steroids are evident in Fig. 17, where their percentage in the mutant cells is determined in relation to the control variant (100%).

Analysis of the relative content of other lipid classes shows a statistically significant decrease in a phosphorus-containing fraction of lipids in some mutants (*nys*^{r} 30–35 and *nys*^{r} 20–25). An increase in 1,2-diacylglycerols (mutants *nys*^{r} 30–35 and *nys*^{r} 20–25) is observed; in lipids of mutant *nys*^{r} 20–25 the content of triacylglycerols is twice as large. The content of fatty acid ethers and steroids in lipids of mutant *nys*^{r} 30–35 is several times higher.

Table II shows data on the content of fatty acids in a total fraction of neutral lipids of nystatin-resistant mutants. It is significant that in the fraction of neutral lipids, fatty acids with a carbon chain length ranging from 14 to 18 atoms are the most cmmon compounds. Oleic acid ($C_{18:1}$) and palmitic acid ($C_{16:0}$) are predominant with respect

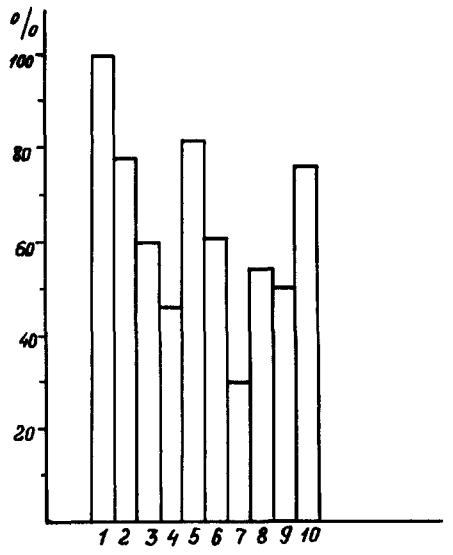


Fig. 17. Graphic representation of relative steroid contents in cells of *Candida tropicalis* D-2 mutants resistant to nystatin: 1 - *nys*^{s}, 2 - *nys*^{r} 7–10, 3 - *nys*^{r} 8–10, 4 - *nys*^{r} 10–12, 5 - *nys*^{r} 20, 6 - *nys*^{r} 30–35, 7 - *nys*^{r} 60, 8 - *nys*^{r} 50–60, 9 - *nys*^{r} 20–25, 10 - *nys*^{r} 80.

to relative amount. The content of various fatty acids in neutral lipids of the initial sensitive strain cells is as follows: lauric acid ($C_{12:0}$), 0.7%; myristic acid ($C_{14:0}$), 0.1%; palmitic acid ($C_{16:0}$), 20.2%; palmitoleic acid ($C_{16:1}$), 32.9%; heptadecanoic acid ($C_{17:0}$), 0.1%; heptadecaenoic acid ($C_{17:1}$), 0.7%; oleic acid ($C_{18:1}$), 37.3%; stearic acid ($C_{18:0}$) 7.0%. Analysis of the fatty acid content of lipids of the mutants established that some differed from the control variant and that some were similar in both qualitative and quantitative aspects. The fatty acid content of a total fraction of neutral lipids of mutant *nys*^{r} 7–10 was analogous to that of a sensitive strain. Lipids of mutant *nys*^{r} 60 differ from those of the initial strain in the relative ratio of saturated and unsaturated fatty acids with a carbon chain length ranging from 16 to 18 atoms.

Noticeable differences in the ratio of these fatty acids have been also found in a fraction of neutral lipids of mutants *nys*^{r} 8–10, *nys*^{r} 20, *nys*^{r} 50–60 and *nys*^{r} 20–25.

Hence, identical classes of neutral lipids as compared with the control ones have been found in all the mutants studies by TLC, but their relative content differed from that of the control sensitive variant. GLC analysis of a neutral lipid fraction also led to the establishment of some differences in the ratio of saturated and unsaturated fatty acids with a different carbon chain length. It should be mentioned that no correlation was found between changes in the relative content of certain fractions of neutral lipids and particular fatty acids, and the level of resistance of *nys*^{r} mutants or their original strain.

Discussion

Considering the results obtainedby electron-microscopic and biochemical studies, the following

TABLE II

FATTY ACID CONTENT OF TOTAL FRACTION OF NEUTRAL LIPIDS OF INDEPENDENTLY OBTAINED *CANDIDA TROPICALIS* D-2 MUTANTS RESISTANT TO NYSTATIN

Length of acid chain	Phenotypes									
	nys^s	*nys*^{r} 7–10	*nys*^{r} 8–10	*nys*^{r} 10–12	*nys*^{r} 20	*nys*^{r} 30–35	*nys*^{r} 60	*nys*^{r} 50–60	*nys*^{r} 20–25	*nys*^{r} 80
$C_{12:0}$	0.7	0.3	0.3	0.9	0.8	0.7	0.5	0.9	0.9	0.3
$C_{14:0}$	0.1	–	–	0.1	0.1	0.1	0.5	0.1	0.1	–
$C_{15:0}$	20.2	22.5	14.4	21.9	22.4	22.4	4.7	13.4	14.2	21.8
$C_{16:0}$	32.9	30.9	26.0	32.0	30.8	31.3	38.7	32.1	30.6	29.8
$C_{17:1}$	0.7	0.8	0.6	0.7	0.7	0.8	0.8	0.9	0.7	0.5
$C_{17:0}$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1
$C_{18:1}$	38.3	38.1	46.0	37.2	35.8	36.8	48.8	44.0	45.5	40.1
$C_{18:0}$	8.0	7.2	12.6	7.1	9.4	7.8	8.9	8.4	7.9	7.4

details should be noted. The organization of ultrathin sections of cells of a polyene-sensitive strain is characteristic of cells carrying out the aerobic type of metabolism [28,29] at the stationary phase of growth in carbohydrate substrates. A relatively small size and regular shape of the nucleus, the presence of a considerable number of small mitochondria and a relatively well developed system of endoplasmic reticulum are characteristic features. However, the considerable thickness of the cell wall and intensive deposition of glycogen granules show the attenuation of active catabolic processes that take place during the stationary phase of growth. Filling of certain vacuoles and phagolysosomes with an electron-dense substrate may be evidence for considerable accumulation of components such as polyphosphates, phosphates, etc. [30,31]. Cells of mutants resistant to nystatin have a considerably greater amount of such components. At the same time they contain less glycogen granules, especially in cells of a mutant with a high degree of resistance.

As compared with the control variant, cells of the strain resistant to nystatin contain microbodies participating in fatty acid metabolism [32,33] and leading to protection of cells from peroxide compounds [34]. Mitochondria in mutants are characterized by a more well developed system of cristae and a part of the organelles is fixed at the moment of division, which shows intensive aerobic metabolism.

It should be mentioned that the clearest changes occur in the cell wall structure. Its extraordinary thickness, the formation of invaginations into cytoplasm and formation of lumps of cell wall substrate show its abnormal synthesis when regulation of deposition in certain vital compartments is disturbed. This synthesis involves membranes of the endoplasmic reticulum which is often arranged along the plasmalemma or in the immediate vicinity thereof. A thicker cell wall may predetermine difficulties in nystatin penetrating into cells which causes their greater resistance to its action.

The changes found in the ratio of different fractions of neutral lipids, and in the fractional decrease of steroids in particular, correspond to data of other authors obtained on yeast-like fungi from the *Candida* genus and on saccharomycetes in which the mechanism of actin of polyene antibiotics consists of interaction with the steroid components of cytoplasmic membranes [5–9]. The following types of changes in steroid components have been revealed in lipids of yeast and yeast-like fungi strains resistant to polyenes: a decrease in the total amount of steroids in cells, a decrease in the total amount of ergosterol only, with the total amount of steroids retained at the level of the wild type; the absence of ergosterol and its substitution by another sterol component. New steroids may be products of biosynthetic by-passes of ergosterol [35,36]. It may be supposed that changes in steroids and in other components of yeast cells lead to disturbance in the adsorption of polyene antibiotics by cells and then to changes in specific complexes of antibiotics with membrane components, which results in breakdown of the selectional permeability of membranes and rearrangement of membrane compartments of the cell.

The changes revealed in content of different fractions of lipids and fatty acids may be due to mutation alterations ingenes responsible for biosynthesis of membrane components other than in those responsible for steroid biosynthesis. Ordinary [36] phenotypic modifications resulting from screening and reorientation of cytoplasmic membranes are also not excluded. An opniion which has also been advanced is that steroid ethers, of which the function in cells has not been well studied [37], are of particular importance for resistance to polyenes because model experiments have demonstrated that steroid ethers do not interact strongly with polyene antibiotics [38–40].

Our data show that the most significant differences in mutants relate to steroids, but there are also changes in other lipid classes. These data and the fact that polyenes may interact with bacterial membranes not containing steroids [41,42], and that there exist mutants and strains resistant to polyene action without any changes in the steroids [43], give reason to suggest that not only steroids are responsible for the resistance to this group of antibiotics. It is necessary to add that polyenes may actively interact with lipids and phospholipids, thus affecting in a certain way steroid-free cells [44,45]. This shows that a 'steroid' hypothesis, completely accounting for the polyene action, may be essentially complemented.

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