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Research article

# THE PHYSIOLOGICAL AND MORPHOLOGICAL PHENOTYPE OF A YEAST MUTANT RESISTANT TO THE QUATERNARY AMMONIUM SALT N-(DODECYLOXYCARBOXYMETHYL)-N,N,N-TRIMETHYL AMMONIUM CHLORIDE

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**Abstract:** We investigated the action of the quaternary ammonium salt (QAS) called IM (N-(dodecyloxycarboxymethyl)-N,N,N-trimethyl ammonium chloride) on *Saccharomyces cerevisiae* yeast cells. Changes in the yeast cell ultrastructure were confirmed by electron microscopy. We treated resistant mutant cells with QAS, and confirmed destruction of the mutant cytoplasm, an increase in the thickness of the cell wall, separation of the cell wall from the cytoplasm, and the accumulation of numerous lipid droplets. We also observed a relatively high production of lipids in the cells of the parental wild-type strain Σ1278b and in its IM-resistant ($IM^R$) mutant in the presence of the QAS. The $IM^R$ mutant showed increased sensitivity to $CaCl_2$ and SDS, and resistance to ethidium bromide, chloramphenicol, erythromycin and osmotic shock. It also tolerated growth at low pH. We suggest that the resistance to IM could be connected with the level of permeability of the cell membrane because the $IM^R$ mutant was sensitive to this compound *in vivo* in the presence of SDS and guanidine hydrochloride, which cause increased permeability of the cell plasma membrane.

**Key words:** Quaternary ammonium salts, Drug resistance, *Saccharomyces cerevisiae*

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Abbreviations used: HBV – human hepatitis B virus; IM – (N- (dodecyloxycarboxymethyl)-N,N,N-trimethyl ammonium chloride), QAS – quaternary ammonium salt

## INTRODUCTION

Quaternary ammonium salts (QAS), especially those of cationic surfactant character, are used as disinfectants, but the detailed mechanism of their antimicrobial activity is far from being understood [1-6]. These drugs are used for a variety of clinical purposes, for example, preoperative disinfection of unbroken skin, application to mucous membranes, and as medicaments [3-5, 7-11]. In addition to having antimicrobial properties, QASs are also excellent for hard-surface cleaning and deodorization [11]. Moreover, QASs are membrane-active agents [12]: their target site is predominantly on the cytoplasmic (inner) membrane of bacteria or the plasma membrane of yeasts [13]. They react with phospholipid components in the cytoplasmic membrane [14], causing membrane distortion and protoplast lysis under osmotic stress. For example, cetylpyridium chloride (CPC) induces the leakage of $K^+$ and pentose material from the yeast *Saccharomyces cerevisiae* and induces protoplast lysis [15]. QASs are sporostatic; they inhibit the outgrowth of spores (the development of a vegetative cell from a germinated spore) but not the actual germination processes (development from dormancy to a metabolically active state). The mechanism of this sporostatic action is unknown [26]. Similarly, QASs are not mycobactericidal, but have a mycobacteriostatic action, although their actual effects on mycobacteria have not been studied in detail [17]. QASs have an effect on lipid-enveloped viruses, including human immunodeficiency virus (HIV) and human hepatitis B virus (HBV), but not on non-enveloped viruses [18, 19]. QAS-based products were found to induce disintegration and morphological changes in human HBV, resulting in a loss of infectivity [20]. In studies with different phages [21, 22], cetylpyridium chloride (CPC) significantly inhibited transduction by the bacteriophage F116 and inactivated the phage particles. Furthermore, CPC altered the protein bands of F116, but did not affect the phage DNA within the capsid.

The extensive use of quaternary ammonium disinfectants in recent years has led to the development of resistance in microorganisms to these drugs. *Staphylococcus aureus* strains contain plasmid-carrying genes encoding resistance to quaternary ammonium compounds and ethidium bromide. The membrane protein QacA (the product of the *qacA* gene) confers multidrug resistance by exporting the compound via the proton motive force, which is generated by the transmembrane electrochemical proton gradient [23].

There is no information about the yeast genes directly involved in the phenotype of resistance to QAS. However, it was shown that triple deletion of the *PDR5*+S*NQ2*+*YOR1* genes caused hypersensitivity of the yeast *Saccharomyces cerevisiae* to tetradecyl trimethyl ammonium bromide, hexadecyl trimetyl ammonium bromide and n-dodecyl trimethyl ammonium bromide [24]. Moreover, the yeast cells were observed to be hypersensitive to QAS in the case of *PDR1* and *PDR3* double-deletion mutants.

Proteins belonging to the pleiotropic drug-resistance family in yeast are able to transport a variety of unrelated compounds upon the hydrolysis of ATP. In the yeast *Saccharomyces cerevisiae*, an over-expressed *PDR5* gene has been shown to cause resistance to a wide range of structurally and functionally unrelated compounds such as antifungals, herbicides, and anticancer and cytotoxic drugs [25]. On the other hand, the over-expression of *CDR1* in *S. cerevisiae* and *Candida albicans* leads to resistance to azole-based antifungal agents [26].

QASs contain a positively charged nitrogen head and a hydrophobic aliphatic carbon chain in their structure. These compounds inhibit the growth of *S. cerevisiae*, and their growth-inhibitory activity is dependent on the aliphatic chain length of the drug. QASs with twelve to fourteen carbon atoms displayed the highest level of biological activity [27, 28]. Sensitivity to QAS also depends on the biosynthetic and respiratory proficiencies of the yeast. Therefore, auxotrophic and respiratory-deficient mutants are more sensitive than isogenic prototrophic and respiratory-proficient strains [27-32]. In previous investigations, it was also found that the inhibitory activity of QAS is pH dependent: the activity of QAS is higher at pH 8 than at pH 6 [27, 28, 33]. Moreover, the compounds inhibit specific and general amino-acid transport into yeast cells [34, 35] and the activity of the plasma membrane and mitochondrial ATPases [27, 28, 34].

In order to more precisely locate the target of QAS action in yeast cells, we analysed the morphological changes occurring under the influence of the tested compound. Having identified the round bodies in the preparations as lipid drops, we performed a comparative quantitative analysis of the lipids and fatty acids.

## MATERIALS AND METHODS

### Chemicals

The structure of the quaternary ammonium salt, compound IM, synthesized at the Department of Chemistry, Technical University of Wroclaw, Poland, is shown in Fig. 1. This drug was obtained by the quaternization of n-dodecyl chloroacetate with trimethylamine in ethereal solution at room temperature [36]. $^1$H-NMR spectra (Bruker instrument 300 MHz, $CDCl_3$, HMS as the internal standard) confirmed the high purity of the synthesized compound. The compound was dissolved in water and added to YPD medium buffered to pH 6 with Sörensen's buffer (0.05 M) to obtain suitable final concentrations.

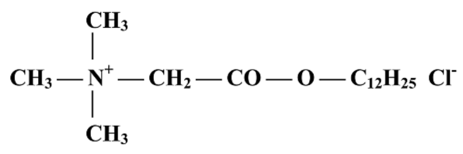


Fig. 1. The chemical structure of the quaternary ammonium salt IM (N-(dodecyloxycarboxymethyl)-N,N,N-trimethyl ammonium chloride).

**Yeast strains and growth conditions**

Two strains of *Saccharomyces cerevisiae* were used in the experiments: the wild-type strain Σ1278b (α prototroph) and its monogenic IM-resistant mutant EO25. The yeast cells were incubated for 7 days on YPD medium (1% Difco yeast extract, 1% Difco bacto peptone, 2% glucose, and 2% Difco bacto agar) with or without IM at pH 6 in final concentrations of 80 or 320 μM. The pH of the medium was adjusted with Sörensen's phosphate buffer (0.05 M).

**Preparation of ultrathin slices for electron microscopy**

Pelleted yeast cells were washed twice with distilled water and fixed in 2.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS) at pH 7.4, rinsed several times in PBS, and fixed with 1% $OsO_4$. After dehydration in increasing concentrations of ethanol and acetone, the cells were embedded in Epone 812. Ultrathin slices were contrasted with uranyl acetate and then with lead citrate according to Reynolds [37]. The material was analyzed under Tesla BS 540 and JEOL 100 transmission electron microscopes.

**Cytochemical analysis**

Yeast cells in suspension were fixed in 4% formalin for 24 h, and immersed first in 15% gelatine for 24 h and then in 30% gelatine for 24 h, both at a temperature of 37ºC. The gelatine blocks with yeast cells were first embedded in fresh gelatine at room temperature, then frozen, and finally sectioned. The sections were stained with Sudan III and photographed under a Nicon Eclipse 50 microscope.

**Analysis of the total lipids and fatty acids**

A wet mass of yeast cells was lyophilised, and the total lipids from 1 g of dry mass were extracted twice by shaking with chloroform/methanol (2:1, v/v) at the ambient temperature for 12 h [38]. The combined chloroform phase from the centrifuged (4000 rpm, 4ºC) suspension was dried for the gravimetric determination of the total lipid weight. Samples for fatty acid analysis were dried over $P_2O_5$ and subjected to acid methanolysis with 1 M HCl in MeOH by the addition of methanol (400 μl) and acetyl chloride (50 μl), and the reaction was performed at 80ºC for 1 h. The solvent was evaporated with a stream of nitrogen and the derived fatty-acid methyl esters were analyzed as described [39] by gas-liquid chromatography combined with mass spectrometry (GLC-MS) using a Hewlett-Packard 5971A system, employing an HP-1 glass capillary column (0.2 mm x 25 m) and a temperature program of 150-270ºC at 8ºC/min. The methyl ester of pentadecanoic acid was used as an internal standard.

**Sensitivity to drugs**

The parental strain Σ1278b and the IM-resistant mutant EO25 were tested for sensitivity to erythromycin (300 μg/ml) and chloramphenicol (2 mg/ml) on YPGly medium (1% Difco yeast extract, 2% Difco bacto peptone, 4% glycerol, and 2% Difco bacto agar). This medium was buffered with 0.1 M $K_2HPO_4$ and adjusted to pH 6.8 prior to autoclaving. The sensitivity of the yeast to other drugs, such as ethidium bromide (25 and 50 μg/ml), SDS (0.05%), and $CaCl_2$ (0.5 M) was investigated on YPD medium. However, the sensitivity levels of both strains to DMSO (7%) were examined on YNB medium (0.67% Difco yeast nitrogen base without amino acid, 2% glucose, 2% Difco bacto agar). The yeast cells were incubated on solid medium in the presence of the drugs at 28ºC for 6 days.

**Measurements of the yeast tolerance to temperature, osmotic shock and low pH**

The thermotolerance experiments were done according to the method of Panaretou *et al.* [40]. The sensitivity of both yeast strains to osmotic shock was tested on YPD medium in the presence of 2 M sorbitol. The yeast cells were incubated in this medium at 28ºC for 6 days. The growth of the wild-type strain and its IM-resistant mutant at low pH was tested on YNB medium. The pH of the medium (5.0, 4.9, 4.8, 4.7) was maintained with an acetate buffer (0.2 M). The growth of the yeast cells in acidic pH was also investigated using YPD medium. Under these conditions, the pH of the medium was adjusted to 4.5 with 1 M HCl and to 7.5 with 1 M KOH. The growth rate was assessed after 6 days.

**The acidification of the medium by the yeast cells**

Yeast cells can secrete acid metabolites into the medium. This phenomenon was examined on CK medium (1% Difco yeast extract, 2% Difco bacto peptone, 0.5% glucose, 2% Difco bacto agar, and the pH indicator bromocresol purple) [41]. The pH of this medium was adjusted to 7.0 with 1 M NaOH. Bromocresol purple is purple at pH 7 and yellow at pH 5. There was an observable change in the colour of the medium from purple to yellow as a pH decrease was caused by the yeast cells acidifying the medium. The ability of the yeast cells to acidify the medium was determined after 2 days of incubation at 28ºC.

**The influence of permeability to IM resistance**

We checked the influence of SDS and guanidine hydrochloride ($CH_5N_3HCl$), a membrane-permeabilizing agent, on the IM resistance of the EO25 mutant and its parental strain. This test was carried out on solid YNB medium at pH 6 in the presence of the compounds at final concentrations of 20 μg/ml SDS, 100 μM guanidine hydrochloride, and 160 or 320 μM IM. The pH of the medium was adjusted with 0.05 M Sörensen's phosphate buffer. The growth of both strains was thus determined on YNB medium alone, on YNB with SDS and guanidine hydrochloride, on YNB with SDS, guanidine hydrochloride and IM, and on YNB with IM. The yeast cells were incubated for 6 days at 28ºC.

## RESULTS

**The effect of IM on cell morphology**

The effect of this QAS on the yeast *Saccharomyces cerevisiae* is visible in the cell morphology as seen in the transmission electron micrographs of the ultrathin sections. To observe the changes caused by IM in the cells of the wild strain and its resistant mutant, the strains were incubated with and without IM at the appropriate minimal inhibitory concentrations (MIC). At concentrations lower than the MIC (80 μM IM for Σ1278b and 320 μM IM for the resistant mutant EO25), such changes were not observed.

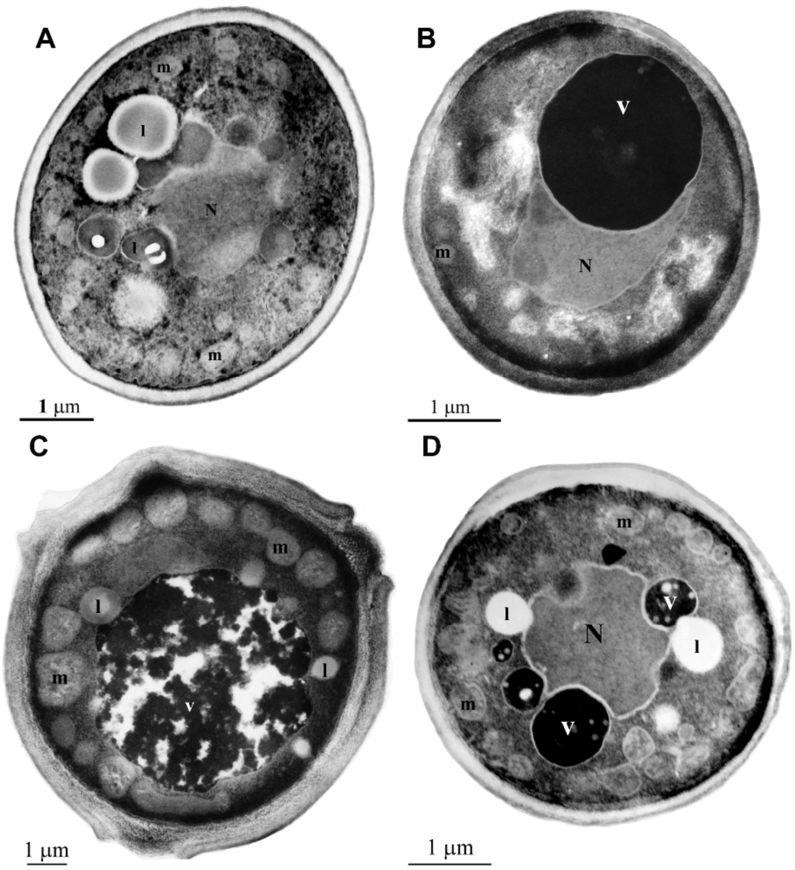


Fig. 2. Transmission electron micrographs of ultrathin sections of the yeast *Saccharomyces cerevisiae* wild-type IM-sensitive strain Σ1278b cultured in YPD medium at pH 6. A – without the IM (control) and B, C, D – in the presence of IM (at a final concentration 80 μM). Legend: l – lipid; v – vacuole; m – mitochondria; N – cell nucleus. Bars = 1μm.

The cells from the control for the sensitive strain Σ1278b (without IM treatment) have a normal oval shape in cross-section. In the cytoplasm, we observed mitochondria, ribosomes, vacuoles with a uniform electron-dense structure, and single lipid droplets (Fig. 2A). By contrast, a slight thickening of the cell wall (Fig. 2B-D) can be observed on the transmission electron micrographs of the QAS-treated cells. In their cytoplasm, there are several mitochondria and ribosomes, and one large vacuole or a few smaller ones, containing regular

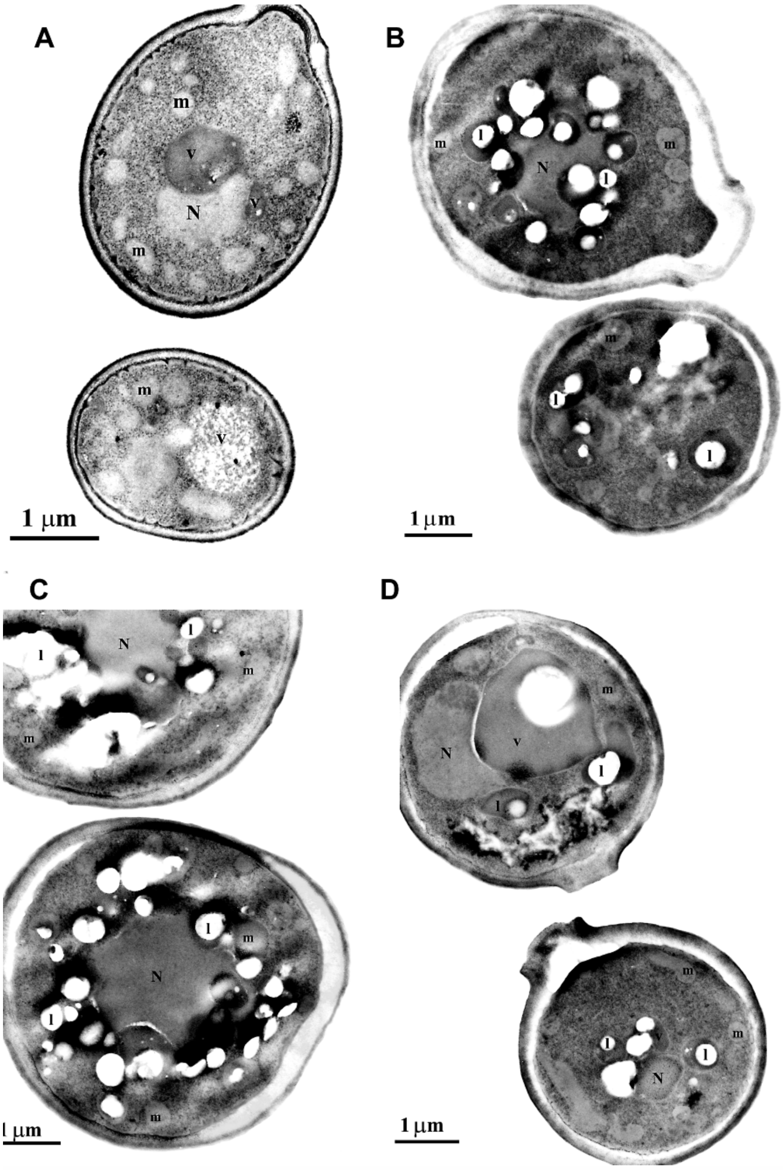


Fig. 3. Transmission electron micrographs of ultrathin sections of the yeast *Saccharomyces cerevisiae* IM-resistant mutant EO25 cultured in YPD medium at pH 6. A – without the IM (control) and B, C, D – in the presence of IM (at a final concentration 320 μM). Legend: l – lipid; v – vacuole; m – mitochondria; N – cell nucleus. Bars = 1μm.

electron-light drops. There are also vacuoles with either a uniformly electron-dense structure or a flocculent structure. In the cells from the control for the IM-resistant mutant EO25, the thickness of the cell wall did not change. The cell nucleus, numerous mitochondria and ribosomes, and single vacuoles were visible in the cytoplasm (Fig. 3A). In the pictures of the cells (Fig. 3B-D) of the mutant EO25 treated with IM, there is a considerable increase in the thickness of the cell wall compared with the control (Fig. 3A). This wall has a spongy character, and there are places where the wall is separated from the cytoplasm. In the cytoplasm, there are also single mitochondria and numerous ribosomes, and the cell nucleus is surrounded by a few lipid droplets.

These results are based on an analysis of 50 ultrathin sections with cell numbers ranging from 60 to 80. We observed a thickening of the cell walls in 70% of the mutant cells and in 30% of the cells of strain Σ1278b.

**The lipid content in whole cells treated with the IM**

To confirm that the yeast cells accumulate lipids in the presence of QAS, both the parental and mutant strains were grown on YPD medium, plated with or without IM, and stained with Sudan III. As shown in Figs 4A and 4B, the cells of both strains acquired an intense red colour in the presence of IM, but remained colourless without IM. The same differences in colour, although not so clear-cut, could be observed between the colonies grown with (Figs 4D and 4F) and without IM (Figs 4C and 4E) after Sudan III treatment of both the sensitive strain and its resistant mutant.

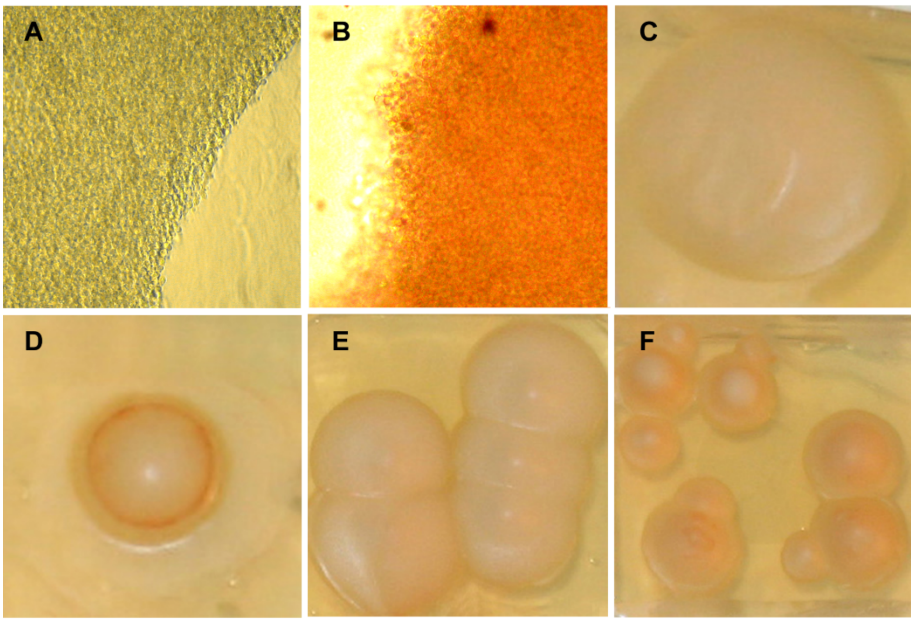


Fig. 4. Ultrathin sections and colonies of the yeast cells stained with Sudan III. A – yeast growing on YPD medium (control); B – yeast growing on YPD medium with IM. Colonies of yeast (the wild-type strain and its IM-resistant mutant) incubated on YPD medium with and without IM were stained with Sudan III. The red colour reveals the presence of lipids. Strains: C – Σ1278b (control); D – Σ1278b in the presence of 80 μM IM; E – EO25 (control); F – EO25 in the presence of 320 μM IM.

**A biochemical analysis of lipids in IM-resistant and sensitive yeast cells**

Further confirmation that the yeast cells accumulate lipids as a result of QAS treatment was obtained when their contents were directly compared. As shown in Tab. 1, the QAS treatment of yeast cells leads to a marked increase in the lipid content, mainly in the saturated fatty acid content in both strains. Under these conditions, the total lipid content in the IM-resistant mutant is considerably higher than in the wild-type parental strain Σ1278b. The resistance to IM causes a relatively higher production of fatty acids, especially saturated ones. However, we did not find any significant difference in the total lipid content between the wild-type strain and its IM-resistant mutant under the control conditions (Tab. 1). There are relatively more unsaturated than saturated fatty acids in both strains.

Tab. 1. An analysis of the total lipids and fatty acids of *Saccharomyces cerevisiae* strains. The values are the means of three experiments, and the standard deviations are given.

| Strain | Total lipids (mg/g dry cell mass) | Saturated fatty acids S (mg/g dry cell mass) | Unsaturated fatty acids NS (mg/g dry cell mass) | Ratio of saturated to unsaturated fatty acids S/NS |
|---|---|---|---|---|
| Σ1278b control | 1.91 ± 0.09 | 0.52 ± 0.05 | 1.39 ± 0.12 | 0.4 |
| Σ1278b + 80 μM IM | 2.69 ± 0.25 | 1.41 ± 0.12 | 1.28 ± 0.07 | 1.1 |
| EO25 control | 1.88 ± 0.17 | 0.67 ± 0.02 | 1.21 ± 0.08 | 0.6 |
| EO25 + 320 μM IM | 3.25 ± 0.06 | 2.10 ± 0.20 | 1.15 ± 0.09 | 1.8 |

**The pleiotropic character of the mutation conferring resistance to IM**

To test whether the resistance mutation phenotype is specific to QAS, the parental strain Σ1278b and the IM-resistant mutant EO25 were tested for their sensitivity to erythromycin (300 μg/ml) and chloramphenicol (2 mg/ml) on YPGly medium, to ethidium bromide (25 and 50 μg/ml), SDS (0.05%) and $CaCl_2$ (0.5 M) on YPD medium, and to DMSO (7%) on YNB medium. These results are shown in Tab. 2 and Fig. 5. As can be seen in Fig. 5, the IM-resistant

Tab. 2. The phenotype of the wild strain and its IM-resistant mutant. The phenotypic tests were repeated at least three times. S – sensitive, R – resistant.

| Strain | Growth in the listed media in the presence of the listed compounds | | | | | | |
|---|---|---|---|---|---|---|---|
| | YPD | | | | YNB | YPGly | |
| | IM 320 μM | EBR 50 μg/ml | $CaCl_2$ 0.5 M | SDS 0.05% | DMSO 7% | ERY 300 μg/ml | CHL 2 mg/ml |
| Σ1278b | S | S | R | R | S | S | S |
| EO25 | R | R | S | S | R | R | R |

YPD medium – 1% Difco yeast extract, 1% Difco bacto peptone, 2% glucose and 2% Difco bacto agar; YNB medium – 0.67% Difco yeast nitrogen base without amino acid, 2% glucose, 2% Difco bacto agar; YPGly medium – 1% Difco yeast extract, 2% Difco bacto peptone, 4% glycerol, and 2% Difco bacto agar

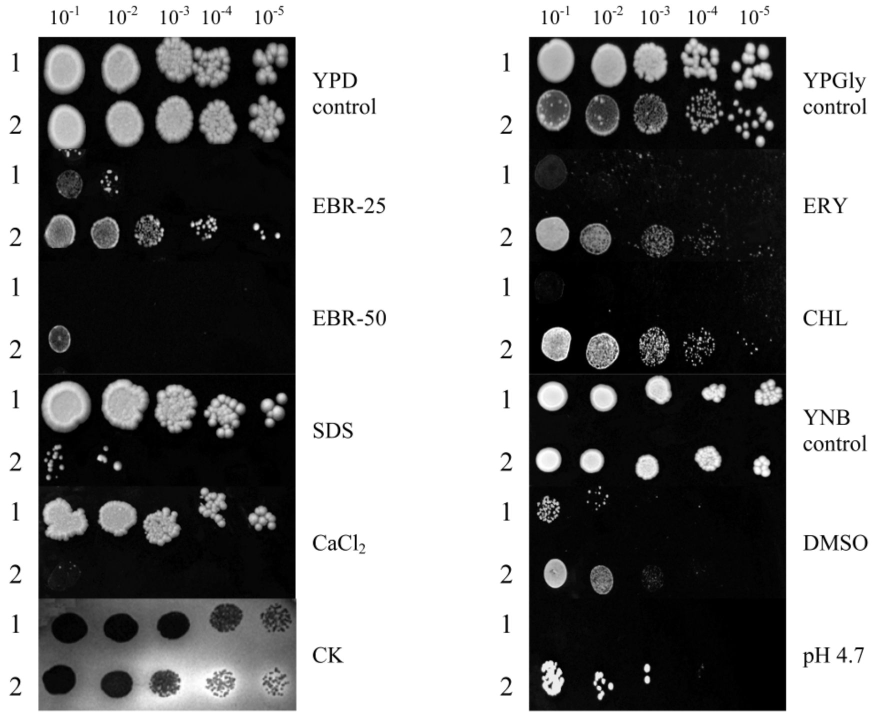


Fig. 5. The sensitivity of the wild-type strain Σ1278b (1) and its IM-resistant mutant EO25 (2) to various growth inhibitors. ERY – erythromycin 300 µg/ml, CHL – chloramphenicol 2 mg/ml, EBR – ethidium bromide 25 and 50 µg/ml, SDS 0.05%, $CaCl_2$ 0.5 M, DMSO 7%. CK – complete medium with bromocresol purple, used for the identification of the acid metabolites secreted by the yeast to the medium, yeast grown on YNB medium with pH 4.7. The phenotypic tests were repeated at least three times.

Tab. 3. The sensitivity of the parental and mutant strains to osmotic shock and to the pH of the medium.

| Strain | Control | Colony forming units (%) in the YPD medium in the presence of sorbitol and at different pH* | | |
|---|---|---|---|---|
| | | Sorbitol 2 M | pH 4.5** | pH 7.5** |
| Σ1278b | 100 | 7.8 | 52 | 58 |
| EO25 | 100 | 84.5 | 83 | 86 |

*Average values are given for tests repeated at least three times. **The pH 4.5 medium was adjusted with 1 M HCl and the pH 7.5 medium with 1 M KOH.

mutant is also resistant to erythromycin, chloramphenicol and DMSO. However, this mutation is associated with some loss of sensitivity to ethidium bromide. Moreover, the IM-resistant mutant acquired sensitivity to $CaCl_2$ and SDS (Fig. 5). It was also noted that the mutant EO25 appears to have an increased resistance to osmotic shock (Tab. 3) and a better tolerance to both the low and alkaline pH of the medium in comparison with the wild-type strain (Fig. 5 and Tab. 3).

It was also demonstrated that the IM-resistant mutant acidifies the medium. This phenomenon was examined using CK medium with a pH indicator (bromocresol purple). The change of colour from purple to a clear yellow zone around the colonies grown on the medium was observed as a result of a decrease in the pH of the medium (Fig. 5). This phenomenon may be associated with the higher secretion of Krebs cycle metabolites by the IM-resistant mutant [41, 42].
Moreover, we observed that resistance to IM increased tolerance to the lethal temperature of 48ºC in cells in which no heat-shock response had been induced (Fig. 6A). This was manifested by a slightly greater survival of the IM-resistant mutant compared with the parental strain. However, after the induction of a heat-shock response and elevated thermotolerance by a shift from 25 to 38ºC followed by incubation of the cells at 38ºC for 40 min, we observed that the IM-resistant mutant showed a lowered tolerance of much higher lethal temperatures (52ºC) than the parental strain (Fig. 6B). The non-acute heat shock at 38ºC enables the induction of HSPs, associated with a marked elevation of thermotolerance. A comparison of Figs 6A and 6B reveals that the $IM^R$ mutation reduces the increase in thermotolerance when the heat-shock response is induced by a shift from 25 to 38ºC. Similar results obtained by Panaretou and Piper [40] indicate that the action of plasma membrane ATPase influences the level of thermotolerance and the extent and duration of heat-shock protein synthesis following induction of the heat-shock response.

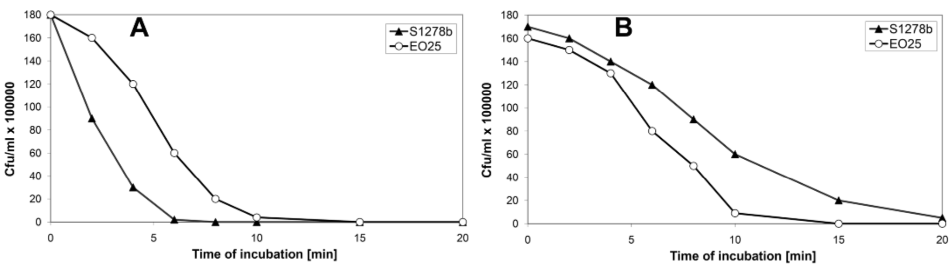


Fig. 6. The high temperature tolerance of the *Saccharomyces cerevisiae* IM-resistant mutant (strain EO25; ○) and its wild-type strain (Σ1278b; ▲). A – The cultures were uninduced for thermotolerance, with a temperature shift from 25 to 48ºC. B – The cultures had their thermotolerance elevated by prior heat shock from 25 to 38ºC for 40 min before being shifted to 52ºC. The average values for two repetitions are given.

One of the effects of heat-shock stress, at least in *Saccharomyces cerevisie*, is dissipation of the transmembrane $H^+$ gradient and a decreased intracellular pH. This intracellular pH drop in turn stimulates plasma membrane ATPase, causing increased proton efflux from the cell, which serves partly to counteract the internal acidification resulting from the stress-induced increase in membrane permeability [43]. The stimulation of plasma membrane ATPase at supra-optimal growth temperatures is not the result of increased biosynthesis of *PMA1* ATPase. This is indicated by the decreased level of transcription of the essential

*PMA1* gene and the corresponding reduction in the level of protein ATPase in the plasma membranes of cells grown at 39ºC compared with cells grown at 30ºC [44, 45]. Similar results were also obtained by Panaretou and Piper [43].
Shin *et al*. [46] reported that a decrease in the cAMP level and the repression of cAMP-dependent protein phosphorylation are required for heat-shock responses in *Saccharomyces cerevisiae*, including the synthesis of hsp proteins, the acquisition of thermotolerance, and the transient arrest of the $G_1$ phase of the cell cycle. Thermotolerance is also affected by the growth state, which is lowest in cells in rapid exponential growth, and high in stationary-phase ($G_0$) cells [47].

**The influence of guanidine hydrochloride (membrane-permeabilizing agent) on IM resistance**

The influence of the QAS on the lipid content in yeast cells and the differences between the parental and mutant strains in this respect permitted us to suppose that the mechanism of this mutation could involve a change in membrane permeability. To verify this supposition, we checked the influence of SDS and guanidine hydrochloride ($CH_5N_3HCl$) on the IM resistance of the EO25 mutant. As seen in Fig. 7, in the presence of SDS and guanidine hydrochloride, EO25 appears to be completely sensitive to IM. Therefore, the permeabilization of the cell membrane eliminated the resistance to IM. We obtained the same results when we tested the resistance to ethidium bromide of both strains (results not shown).

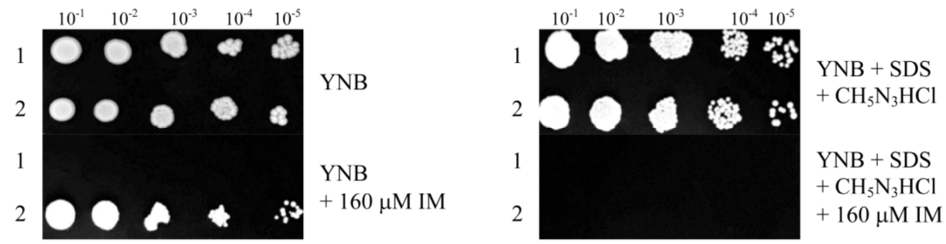


Fig. 7. The sensitivity of the yeast (the wild-type strain and its IM-resistant mutant) to IM in the presence of SDS and hydrochloride guanidine in the YNB medium. Strains: 1 – Σ1278b $IM^S$, 2 – EO25 $IM^R$. The phenotypic test was repeated at least three times.

## DISCUSSION

The antimicrobial activity of synthetic QAS is well known, and these compounds are extensively used as disinfectants [1-6]. QASs contain a positively charged nitrogen head and a hydrophobic aliphatic carbon chain in their structure. They anchor to the lipid regions of the cell membrane by the hydrophobic aliphatic carbon chain. However, their positively charged nitrogen head remains at the surface, thus interfering with its function of proton extrusion by plasma membrane $H^+$-ATPase, especially in the transport of amino acids into the cell [27, 28, 34, 35].

The antibacterial and antifungal activities of QASs depend on the cell wall structure of the microorganism. Quaternary compounds are effective on both Gram-positive and Gram-negative bacteria, but they have a stronger antibacterial effect on Gram-positive bacteria, since Gram-negative bacteria have an extra protective membrane [48-50]. This was shown on spheroplasts and on whole cells of *Pseudomonas aeruginosa* [51].

The actions of various quaternary compounds on yeast cells were also studied. It was demonstrated that QASs and their derivatives were fungicidal toward *Candida species* and *Saccharomyces cerevisiae* and fungistatic toward *Aspergillus ochraceus* conidia [52, 53]. Moreover, investigations by Shirai *et al.* [53] showed (on scanning and transmission electron micrographs) a preserved yeast cell wall structure, whereas intracellular organelles were destroyed in cells incubated in the presence of the compound.

However, in our study, screening the cytoplasm profiles of the IM-resistant mutant originating from Σ1278b revealed destruction of the mutant cytoplasm (in the presence of IM), an increase in thickness of the cell wall, its separation from the cytoplasm, and the accumulation of numerous lipid droplets around the cell nucleus. This cannot be seen in the sensitive strain and in the cells which were not treated with this compound. The observed changes are different from those demonstrated by Shirai [53]. Since we identified the round bodies in the preparations as drops of lipids, a comparative quantitative analysis of the lipids and fatty acids was done. Our investigation confirmed an overproduction of lipids with saturated fatty acids in the cells of both the wild-type (Σ1278b) and IM-resistant mutant (EO25) in the presence of the IM salt.

It is know that the unsaturated fatty acids that play important roles in yeast physiology (e.g. membrane integrity) and biotechnology (e.g. ethanol tolerance) include palmitoleic (16:1) and oleic (18:1) acids. Together, these compounds constitute the bulk (approx. 70%) of the fatty acids in the *Saccharomyces cerevisiae* cell membrane. The others are mainly saturated fatty acids, primarily palmitic (16:0) and lesser amounts of stearic (18:0) and myristic (14:0) acids [54, 55]. The plasma membrane permeability of yeast depends on the amount and type of fatty-acid chains in the phospholipid bilayer. The increased level of unsaturated fatty acids in the plasma membrane increases its permeability, which was also shown in our experiments. The sensitive parental strain (Σ1278b) has slightly more unsaturated fatty acids, and this may be associated with higher amounts of the drug penetrating into the cell, resulting in its growth inhibition. However, the mutant strain has a low amount of unsaturated fatty acids, which results in hampered drug permeation. We suggest that resistance to the quaternary ammonium salt IM can be connected with the degree of permeability of the cell membrane because the IM-resistant mutant is sensitive *in vivo* to this compound in the presence of SDS and guanidine hydrochloride, which cause an increase in the permeability of the plasma membrane [56].

The adaptation of microorganisms to various environmental factors (e.g. antimicrobial compounds) often leads to large chemical changes in their cell surface structure [57, 58]. In solution, the ammonium salt dissociates and interacts with polar cell components and finally changes membrane fluidity and permeability. The resistance to amphiphiles is accompanied by changes in the composition of the cellular outer layers. The mechanism of amphiphile resistance was shown to be the reduction of the negative surface charge by modification of the bacterial cell envelope (LPS and phospholipids), which repels these compounds [58].

Pleiotropic drug resistance in the yeast *Saccharomyces cerevisiae* results mainly from the over-expression of genes encoding membrane efflux pumps, the so-called ABC (ATP-Binding Cassette) and MFS (Major Facilitator Superfamily) transporters [55]. The ABC transporters mediated resistance to most currently available classes of clinically and agriculturally important fungicides and also to many antibiotics, herbicides and anticancer and cytotoxic drugs, and to several membrane lipids resembling detergents and lysosomotropic aminoesters [24, 25]. In our study, we observed that the IM-resistant mutant is resistant not only to QAS but also to chloramphenicol, erythromycin and ethidium bromide, which are effluxed by transporters controlled by the Pdr1p and Pdr3p transcription factors [24, 25, 59]. This raises the possibility that the mutant is modified in the energy pathways required to activate these pumps.

It was demonstrated that the *YOR1* gene product is the major resistance factor to erythromycin, and that its deletion causes a slight increase in sensitivity to QAS (n-dodecyl trimethyl ammonium bromide) [24]. However, hypersensitivity was observed in *PDR5+SNQ2+YOR1* triple deletion and *PDR1+PDR3* double-deletion mutants.

Moreover, it was shown that the modification of the ABC transporters affects the sterol and phospholipid composition of the plasma membrane, and that they change the total yeast lipid composition [60, 61]. Thus, in response to the application QASs as disinfectants, microorganisms acquired different mechanisms of resistance, such as the reduction of drug import into the cells and/or the active efflux of drug from the cell (e.g. the efflux pumps ABC and MFS). To prevent drugs from entering, cells can alter the composition of their membrane bilayer, for example the proportions of sterols (ergosterol) and phospholipids, which are two major components of the fungal plasma membrane [49, 50]. Their interaction regulates the fluidity and asymmetry of the membrane, which control the transport of materials across it. There are also other mechanisms of drug resistance, such as altering the drug target, including mutations of the target, over-expressing the target, and changing other enzymes in the same enzymatic pathway as the drug-targeted enzyme. Moreover, drug resistance can be caused by inactivation of the drug after its penetration into the cell, including its modification and degradation by engaging in the metabolic cycles of the cell [49, 50]. The cloning of genes conferring resistance to QAS is in progress.

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## REFERENCES

1. Xiao, Y., Chen, J., Fang, M., Xing, X., Wang, H., Wang, Y. and Li, F. Antibacterial effects of three experimental quaternary ammonium salt (QAS) monomers on bacteria associated with oral infections. **J. Oral Sci.** 50 (2008) 323-327.
2. Thorsteinsson, T., Masson, M., Kristinsson, K.G., Hjalmarsdottir, M.A., Hilmarsson, H. and Loftsson, T. Soft antimicrobial agents: Synthesis and activity of labile environmentally friendly long chain quaternary ammonium compounds. **J. Med.** 46 (2003) 4173-4181.
3. Massi, L., Guittard, F. and Geribaldi, S. Quaternary bisammonium fluorosurfactants for antimicrobial devices, **Progr. Colloid Polymer Sci.** 124 (2004) 190-193.
4. Mc Donnell, G. and Russell, A.D. Antiseptics and disinfectants activity, action and resistance. **Clin. Microbiol. Rev.** 12 (1999) 147-179.
5. Kourai, H., Yabuhara, T., Shirai, A., Maeda, T. and Nagamune, H. Syntheses anitimicrobial activities of a series of new bis-quaternaty ammonium compounds. **Eur. J. Med. Chem.** 41 (2006) 437-444.
6. Denyer, S.P. Mechanism of action of antibacterial biocides. **Int. Biodeterior. Biodegrad.** 36 (1995) 227-245.
7. Ohkawa, K., Kim, H. and Lee, K. Biodegradation of electrospun poly (e-caprolactone) non-woven fabrics by pure-cultured soil filamentous fungi. **J. Polym. Env.** 12 (2004) 211-218.
8. Lee, C. Structure, conformation and action of neuromuscular blocking drugs. **Br. J. Anaesth.** 8 (2001) 7755-7769.
9. Koyama, K. and Shimazu, Y. Benzalkonium chlorides. **Drugs and Poisons in Humans** 23 (2005) 407-413.
10. Debbash, C., de Saint Jean, M., Pisella, P.J., Warnet, J. and Baudouin, C. Quaternary ammonium cytotoxity in a human conjunctinal cell line. **J. Fr. Ophtalmol.** 22 (1999) 950-958.
11. Petrocci, A.N. Surface-active agents: quaternary ammonium compounds. In: **Disinfection, sterilization and preservation**. (Block, S.S. Ed.) Lea and Febiger. Philadelpha. 1983, 309-329.
12. Hugo, W.B. and Frier, M. Mode of action of the antibacterial compound dequalinium acetate. **Appl. Microbiol.** 17 (1969) 118-127.
13. Hugo, W.B. Disinfection mechanisms. In: **Principles and practice of disinfection, preservation and sterilization**, 3rd ed., (Russell, A.D., Hugo, W.B. & Ayliffe, G.A.J., Eds), Blackwell Science, Oxford. 1999, 258-283.
14. Cabral, J.P.S. Mode of antibacterial action of dodine (dodecylguanidine monoacetate) in *Pseudomonas syringae*. **Can. J. Microbiol.** 38 (1991) 115-123.

15. Hiom, S.J., Furr, J.R., Russell, A.D. and Dickinson, J.R. Effects of chlorhexidine diacetate and cetylpyridinium chloride on whole cells and protoplasts of *Saccharomyces cerevisiae*. **Microbios** 74 (1993) 111-120.
16. Russell, A.D. Bacterial spores and chemical sporicidal agents. **Clin. Microbiol. Rev.** 3 (1990) 99-119.
17. Russell, A.D. Activity of biocides against mycobacteria. **J. Appl. Bacteriol. Symp. Suppl.** 81 (1996) 87S-101S.
18. Springthorpe, V.S., Grenier, J.L., Lloyd-Evans, N. and Sattar, S.A. Chemical disinfection of human rotaviruses: efficacy of commercially-available products in suspension tests. **J. Hyg.** 97 (1986) 139-161.
19. Springthorpe, V.S. and Satter, S.A. Chemical disinfection of virus-contaminated surfaces. **Crit. Rev. Environ. Control** 20 (1990) 169-229.
20. Prince, D.L., Prince, H.N., Thraenhart, O., Muchmore, E., Bonder, E. and Pugh, J. Methodological approaches to disinfection of human hepatitis B virus. **J. Clin. Microbiol.** 31 (1993) 3296-3304.
21. Maillard, J.Y. Mechanisms of viricidal action, In: **Principles and Practice of Disinfection, Preservation and Sterilization.** (Russell, A.D., Hugo, W.B., Ayliffe, G.A.J. Eds.), Blackwell Science, Oxford, 1999, 207-221.
22. Maillard, J.Y., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. Damage to *Pseudomonas aeruginosa* PAO1 bacteriophage F116 DNA by biocides. **J. Appl. Bacteriol.** 80 (1996) 540-554.
23. Mitchell, B.A., Paulsen, I.T., Brown, M.H. and Skurray, R.A**.** Bioenergetics of the staphylococcal multidrug export protein QacA: identification of distinct binding sites for monovalent and divalent cations. **J. Biol. Chem.** 274 (1999) 3541-3548.
24. Rogers, B., Decottignies, A., Kolaczkowski, M., Carvajal, E., Balzi, E. and Goffeau, A. The pleitropic drug ABC transporters from *Saccharomyces cerevisiae***. J. Mol. Microbiol. Biotechnol.** 3 (2001) 207-214.
25. Kolaczkowski, M., Kolaczkowska, A., Łuczynski, J., Witek, S. and Goffeau, A. *In vivo* characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. **Microb. Drug Resist.** 4 (1998) 143-158.
26. Yang, Y.L. and Lo, H.J. Mechanisms of antifungal agent resistance. **J. Microbiol. Immunol. Infect.** 34 (2001) 79-86.
27. Obłąk, E., Lachowicz, T.M., Łuczyński, J. and Witek, S. Comparative studies of biological activities of the lysosomotropic aminoesters and quaternary ammonium salts on yeast *Saccharomyces cerevisiae*. **Cell. Mol. Biol. Lett.** 6 (2001) 871- 880.
28. Obłąk, E., Lachowicz, T.M., Łuczyński, J. and Witek, S. Lysosomotropic N,N-dimethyl α-aminoacid n-alkylesters and their quaternary ammonium salts as plasma membrane and mitochondrial ATPases inhibitors. **Cell. Mol. Biol. Lett.** 7 (2002) 1121-1129.

29. Lachowicz, T.M., Witkowska, R. and Obłąk, E. Amino acid auxotrophy increases sensitivity of *Saccharomyces cerevisiae* to a quaternary ammonium salt IM. **Acta Microbiol. Polon.** 39 (1990) 157-162.
30. Lachowicz, T.M., Obłąk, E. and Piątkowski, J. Auxotrophy stimulated sensitivity to quaternary ammonium salts and its relation to active transport in yeast. **Bul. Pol. Acad. Sci. Biol. Sci.** 40 (1992) 173-182.
31. Obłąk, E., Ułaszewski, S., Morawiecki, A., Witek, S., Witkowska, R., Majcher, K. and Lachowicz, T.M. Quaternary ammonium salt resistant mutants in yeast *Saccharomyces cerevisiae.* **Yeast** 5 Spec. Iss. (1989) 273-278.
32. Obłąk, E., Ułaszewski, S. and Lachowicz, T.M. Mutants of *Saccharomyces cerevisiae* resistant to a quaternary ammonium salt. **Acta Microbiol. Polon.** 37 (1988) 261-269.
33. Obłąk, E., Adamski, R. and Lachowicz, T.M. pH-dependent influence of a quaternary ammonium salt and an aminoester on the yeast *Saccharomyces cerevisiae* ultrastructure. **Cell. Mol. Biol. Lett.** 8 (2003) 105-110.
34. Obłąk, E., Bącal, J. and Lachowicz, T.M. A quaternary ammonium salt as an inhibitor of plasma membrane $H^+$-ATPase in yeast *Saccharomyces cerevisiae*. **Cell. Mol. Biol. Lett.** 5 (2000) 315-324.
35. Obłąk, E., Lachowicz, T.M. and Witek, S. D,L-leucine transport in a *Saccharomyces cerevisiae* mutant resistant to quaternary ammonium salts. **Folia Microbiol.** 41 (1996) 116-119.
36. Rucka, M., Oświęcimska, M. and Witek, S. New biocides for cooling water treatment. II. Quaternary ammonium salts derivatives of glycine esters. **Envir. Protec. Eng.** 9 (1983) 25-31.
37. Reynolds, E.W. The use of lead citrate at high pH as an electron – opaque stain electron microscopy. **J. Cell. Biol.** 17 (1963) 208-212.
38. Kates, M. **Laboratory Techniques in Biochemistry and Molecular Biology.** Elsevier, Amsterdam. Vol. 3, part 2, 1986, 106-107.
39. Paściak, M., Ekiel, I., Grzegorzewicz, A., Mordarska, H. and Gamian, A. Structure of the major glycolipid from *Rothia dentocariosa*. **Biochim. Biophys. Acta** 1594 (2002) 199-205.
40. Panaretou, B. and Piper, P.W. Plasma membrane ATPase action affects several stress tolerances of *Saccharomyces cerevisiae* and *Schizozaccharomyces pombe* as well as the extent and duration of the heat shock response. **J. Gen. Microbiol.** 136 (1990) 1763-1770.
41. Ogur, M., Roshanmanesh, A. and Ogur, S. Tricarboxylic acid cycle mutants in *Saccharomyces cerevisiae*. Comparison of independently derived mutants. **Science** 147 (1965) 1590.
42. Machnicka, B., Grochowalska, R., Boniewska-Biernacka, E., Słomińska, L. and Lachowicz, T.M. Acid excreting mutants of yeast *Saccharomyces cerevisiae.* **Biochim. Biophys. Res. Commun.** 325 (2004) 1030-1036.
43. Panaretou, B. and Piper, P.W. The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton pumping

ATPase levels in response to both heat-shock and the entry to stationary phase. **Eur. J. Biochem.** 206 (1992) 635-640.
44. Viegas, C.A., Sebastiao, P.B., Nunes, A.G. and Sacorreia, I. Activation of plasma membrane H+-ATPase and expression of PMA1 and PMA2 genes in *Saccharomyces cerevisiae* cells grown at supraoptimal temperatures. **Appl. Environ. Microbiol.** 61 (1995) 1904-1909.
45. Coote, P.J., Jones, M.V., Seymour I.J., Rowe, D.L., Ferdinando, D.P., McArtur, A.J. and Cole, M.B. Activity of plasma membrane $H^+$-ATPase is a key physiological determinant of thermotolerance in *Saccharomyces cerevisiae*. **Microbiology** 140 (1994) 1881-1890.
46. Shin, D.Y., Matsumoto, K., Iida, H., Uno, I. and Ishikawa, T. Heat shock response of *Saccharomyces cerevisiae* mutants altered in cyclic AMP-dependent protein phosphorylation. **Mol. Cell. Biol.** 7 **(**1987) 244-250.
47. Iida, H. and Yahara, Y. Durable synthesis of high molecular weight heat shock proteins in $G_o$ cells of yeast and other eukaryotes. **J. Cell. Biol.** 99 (1984) 199-207.
48. Russell, A.D. Mechanisms of bacterial resistance to biocides. **Int. Biodeterior. Biodegrad.** 36 (1995) 247-265.
49. Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. **Science** 264 (1994) 382-388.
50. Ghannoum, M.A. and Rice, L.B. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. **Clin. Microbiol. Rev.** 12 (1999) 501-517.
51. Guérin-Méchin, L., Leveau, J.Y. and Dubois-Brissonnet, F. Resistance of spheroplasts and whole cells of Pseudomonas aeruginosa to bactericidal activity of various biocides: evidence of the membrane implication. **Microbiol. Res.** 159 (2004) 51-57.
52. Gupta, A.K., Ahmad, I. and Summerbell, R.C. Fungicidal activities of commonly used disinfectants and antifungal pharmaceutical spray preparations against clinical strains of Aspergillus and Candida species. **Med. Mycol.** 40 (2002) 201-208.
53. Shirai, A., Sumitomo, T., Kurimoto, M., Maseda, H. and Kourai, H. The mode of the antifungal activity of gemini-pyridinium salt against yeast. **Biocontrol. Sci.** 14 (2009) 13-20.
54. Walker, G.M. **Yeast physiology and biotechnology.** John Wiley & Sons Ltd. Chichester, England, 1998, 169-231.
55. Van der Rest, M.E., Kamminga, A.H., Nakano, A., Anraku, Y., Poolman, B., and Konings, W.N. The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis. **Microbiol. Rev.** 59 (1995) 304-322.
56. Baranowska, H., Polanowska, R. and Putrament, A. Spontaneous and induced non-specific drug resistance in *Saccharomyces cerevisia*. **Acta Microb. Polon.** 23 (1979) 181-201.

57. Dubnicková, M., Rezanka, T. and Koscová, H. Adaptive changes in fatty acids of E. coli strains exposed to a quaternary ammonium salt and an amine oxide. **Folia Microbiol.** 51 (2006) 371-374.
58. Guérin-Méchin, L., Dubois-Brissonnet, F., Hedyd, B. and Leveau, J.Y. Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. **J. Appl. Microbiol.** 87 (1999) 735-742.
59. Kolaczkowska, A., Kolaczkowski, M., Goffeau, A. and Moye-Rowley, W.S. Compensatory activation of the multidrug transporters Pdr5p, Sng2p, and Yor1p by Pdr1 in *Saccharomyces cerevisiae.* **FEBS Lett.** 582 (2008) 977-983.
60. Van den Hazel, H.B., Pichler, H., do Valle Matta, M.A., Leitner, E., Goffeau, A. and Daum, G. PDR16 and PDR17, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. **J. Biol. Chem.** 274 (1999) 1934-1941.
61. Hallstrom, T.C., Lambert, L., Schorling, L., Balzi, E., Goffeau, A. and Moye-Rowley, W.S. Coordinate control of sphingo lipid biosynthesis and multidrug resistance in *Saccharomyces cerevisiae***.** **J. Biol. Chem.** 276 (2001) 23674-23680.