Quaternary ammonium salt *N*-(dodecyloxycarboxymethyl)-*N*,*N*,*N*-trimethyl ammonium chloride induced alterations in *Saccharomyces cerevisiae* physiology

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We investigated the influence of the quaternary ammonium salt (QAS) called IM (*N*-(dodecyloxycarboxymethyl)-*N*,*N*,*N*-trimethyl ammonium chloride) on yeast cells of the parental strain and the IM-resistant mutant (EO25 IM^R) growth. The phenotype of this mutant was pleiotropic. The IM^R mutant exhibited resistance to ethanol, osmotic shock and oxidative stress, as well as increased sensitivity to UV. Moreover, it was noted that mutant EO25 appears to have an increased resistance to clotrimazole, ketoconazole, fluconazole, nystatin and cycloheximide. It also tolerated growth in the presence of crystal violet, DTT and metals (selenium, tin, arsenic). It was shown that the presence of IM decreased ergosterol level in mutant plasma membrane and increased its unsaturation. These results indicate changes in the cell lipid composition. Western blot analysis showed the induction of Pma1 level by IM. RT-PCR revealed an increased *PMA1* expression after IM treatment.

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1. Introduction

Quaternary ammonium salts (QASs) are common in nature. They are synthesized by organisms for better fitness to environmental conditions, e.g., temperature or salinity (Anthoni *et al*. 1991). QASs participate in different processes, e.g., act as carriers of activated fatty acids across the inner mitochondrial membrane, or as inhibitors of mitotic cell divisions. These compounds exhibit antibacterial activity, thus they are commonly used in medicine as disinfectants (e.g., benzalkonium chloride) (Thorsteinsson *et al.* 2003; Massi *et al*. 2004). QASs are also active against enveloped viruses like HIV (*human immunodeficiency virus*) and HBV (*hepatitis B virus)*, but not nonenveloped viruses (Resnick *et al.* 1986; Springthorpe and Satter 1990). Drugs based on quaternary ammonium salts cause morphological changes in human HBV structure, resulting in the loss of virulence (Prince *et al.* 1993). Numerous compounds of this group are used in anesthesiology as muscle relaxants (Lee 2001). Another QAS - methacryloxylethyl cetyl ammonium chloride (DMAE-CB), exhibited activity against cariogenic and virulent bacterial strains, thus it is being tested as a potential additive to dental materials (Xiao *et al.* 2008). QASs are also applied in the treatment of diabetes, arrhythmia, and neurosis, and they enhance the effectiveness of anticancer drugs. Some data indicate that derivatives with quaternary ammonium ion are more active against cancer cells than their parental compounds, which suggests their potential application in anticancer therapy. These compounds interact with mitochondrial functioning and induce programmed cell death (Ito *et al.* 2009). Another study showed the higher effectiveness of chemotherapeutics when combined with quaternary ammonium ions. Such combination with chlorambucil or melphalan (popular chemotherapeutics) increases the drug's lipophilicity and its ability to penetrate the plasma and mitochondrial membrane (Giraud *et al.* 2002). It was

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also shown that complexes of quaternary ammonium salts and metals (cobalt, copper) have a direct impact on cancer cells by damaging their DNA (Badawi *et al.* 2007). Moreover, it was proved that N,N,N-trimethyl chitosan (TC) exhibits scavenging activity against superoxide anion radicals, whose level rises during aerobic metabolism (Zhu *et al.* 2004). QASs are used as biocides, fungicides, herbicides and insecticides (Hegstad *et al.* 2010). They are applied in industry for biofilm eradication in cooling systems (Pico *et al.* 2000).

Nowadays, the appearance of mutants resistant to disinfectants is an important issue. Intrinsic resistance to QAS is common among Gram-negative bacteria (e.g., *Pseudomonas aeruginosa, Escherichia coli*), mycobacteria and spores (Guerin-Mechin *et al.* 2004; Bore *et al.* 2007). One of the most common mechanisms of resistance is the active efflux of various xenobiotics by membrane transporters ABC (ATP-Binding Cassette) and MFS (Major Facilitator Superfamily), e.g., QAS resistance in *Staphylococcus aureus* is mediated by the enhanced expression of *qacA* and *qacB* genes, encoding efflux pumps (MFS) (Vali *et al.* 2008). On the other hand, the mechanism of QAS resistance among yeast remains unknown.

Our results showed that quaternary ammonium salt *N*-(*N*-dodecyloxycarbonyl)-methyl-*N*,*N*,*N*-trimethyl ammonium chloride (IM) inhibits the growth of Gram-positive and Gram-negative bacteria, *Candida albicans* and *Saccharomyces cerevisiae*. Moreover, a cytotoxicity test (MTT) showed the high IM sensitivity of melanoma cells. A lower ATP level was observed in melanoma cells after exposition to IM, which indicates the activity of this compound against mitochondrial metabolism (Obłąk and Krasowska 2010). Auxotrophic yeast strains exhibited a much higher sensitivity to QASs than prototrophs (Obłąk *et al*. 1989; Lachowicz *et al.* 1990). Similarly, *rho*$^-$ and *rho*^{o} mutants were more sensitive to these compounds than *rho*$^+$ strains (Lachowicz *et al.* 1992; Obłąk *et al*. 1989, 2002, 2004; Obłąk and Krasowska 2010). The activity of QASs against yeast depends on a medium pH; lower inhibitory concentrations were observed for pH 8 than pH 6 (Obłąk *et al.* 2001, 2002, 2003).

Micrographs from a transmission electron microscope showed that QASs influence yeast plasma membrane (Obłąk *et al.* 2003, 2010). These compounds are inhibitors of the plasma and mitochondrial membrane H^+-ATPase of *S. cerevisiae* (*in vitro* and *in vivo*), as well as amino acid transport into the cell (Lachowicz *et al.* 1992, 1995; Obłąk *et al.* 1996, 2000, 2002; Witek *et al.* 1997).

To study the mechanism of resistance to QAS, spontaneous IM-resistant yeast mutants were obtained in the prototrophic wild-type strain Σ1278b (Obłąk *et al.* 1988). The study results showed that resistance to IM was caused by a single nuclear gene mutation segregating in meiosis. All of the tested mutants were allelic and they exhibited a pleiotropic phenotype (Obłąk *et al.* 2000, 2010).

Our study showed that IM-resistant strain became sensitive to IM in the presence of SDS and aminoguanidine hydrochloride (compounds that increase plasma membrane permeability). These results might suggest that IM resistance could be connected with the degree of cell membrane permeability (Obłąk *et al.* 2010). Similar observations were made for QASs resistance in some bacterial strains being the result of changes in cell surface properties (permeability of outer membrane, cell surface charge and hydrophobicity) (Braoudaki and Hilton 2005).

Ergosterol is one of the cell lipids responsible, among other things, for tightening plasma membrane. Our previous results suggested that IM changed the lipid composition in yeast cells, thus in this work we investigated ergosterol and fatty acid content in the plasma membrane of the parental strain and the IM-resistant mutant in the presence of the IM (Obłąk *et al.* 2010). The phenotype of IM^R mutant was similar to *pma1* mutants (Panaretou and Piper 1990), thus we studied Pma1 level and *PMA1* expression in cells of the wild-type strain and mutant EO25 without and in the presence of the IM.

2. Materials and methods

2.1 *Chemicals*

The structure of the quaternary ammonium salt, compound IM, synthesized at the Department of Chemistry, Technical University of Wroclaw, Poland, is shown in figure 1.

This drug was obtained by the quaternization of *n*-dodecyl chloroacetate with trimethylamine in ethereal solution at room temperature (Rucka *et al.* 1983). ^{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 a YPD medium buffered to pH 6 with Sörensen's buffer (0.05 M), to obtain suitable final concentrations.

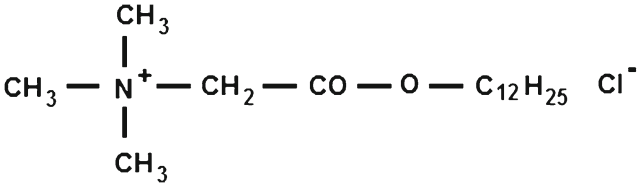

Figure 1. The chemical structure of the quaternary ammonium salt IM(*N*-(dodecyloxycarboxymethyl)-*N*,*N*,*N*-trimethyl ammonium chloride).

2.2 *Yeast strains*

Two strains of *Saccharomyces cerevisiae* were used in the experiments: the parental strain ATCC 42800 Σ1278b (α prototroph) and its derivative IM-resistant mutant EO25 (Obłąk *et al.* 1988).

2.3 *Sensitivity to drugs*

The parental strain Σ1278b and the IM-resistant mutant EO25 were tested for sensitivity to antifungal drugs (ketoconazole (10 μg/mL), clotrimazole (100 μM), fluconazole (60 μg/mL), nystatin (2.4 μg/mL), metals (sodium selenite (8 mM), tin chloride (5 mM), sodium arsenite (0.75 mM)), DNA damaging agents (hydroxyurea (80 mM), 5-fluorouracil (60 μg/mL), 4-nitroquinoline 1-oxide (0.2 μg/mL), methyl methanesulfonate (0.02%)) and other growth inhibitors (cycloheximide (90 ng/mL), crystal violet (30 μg/mL), ditiotreitol (20 mM)) in YPG medium (1% Difco yeast extract, 1% Difco bacto peptone, 2% glucose and 2% Difco bacto agar).

The yeast strains were incubated in YPG medium to obtain 1×10^8 cells/mL and spotted (3 μL) from 10^{-1}, 10^{-2} and 10^{-3} dilutions on a solid medium with the addition of drugs. Plates were incubated at 28°C up to 6 days. This medium was buffered with 0.1 M K_2HPO_4 and adjusted to pH 6.8 prior to autoclaving.

2.4 *Influence of IM on yeast sensitivity to azoles*

To determine the decrease in yeast growth in the presence of azoles caused by IM, both yeast strains were diluted (OD 0.15) in YPG on microtiter plates. Compounds: IM (50 μM and 100 μM), fluconazole (0.3 mg/mL) and itraconazole (10 μg/mL) separately, and IM in combination with fluconazole or itraconazole were added. For IM^R mutant additional higher concentration of IM (100 μM) was used to obtain a comparable effect for the wild type. Cells were incubated at 28°C for 24 h and spread on YPG plates. After 48 h colony forming units (CFU) were counted and CFU log reduction was estimated from the CFU ratio between untreated cells and cells treated with the compounds. Strains incubated without drugs were used as growth controls. This test was repeated three times.

2.5 *Measurements of yeast tolerance to stress*

Tolerance to high ethanol and salt was measured as described before (Panaretou and Piper 1990). Cells in exponential growth ($0.5–1\times10^7$ cells/mL) at 25°C in YEPD (1% Difco yeast extract, 2% Difco bacto peptone, 2% glucose) were diluted in YEPD at 25°C, and 96% ethanol or 4 M NaCl to final concentrations of 12.5% v/v and 2.5 M, respectively, were added. Aliquots of 0.1 mL were removed immediately (zero time point) at subsequent intervals (diluted 100 to 1000-fold in YEPD prior to plating on YEPD). Survival was measured from colony numbers on YEPD plates incubated at 28°C for up to 6 days. Each experiment was repeated at least 3 times, with similar results.

For measurements of UV sensitivity, cells were diluted in YEPD and 0.15 mL aliquots were spread on YEPD plates to obtain 300 cells per plate according to Panaretou and Piper (1990). Immediately after plating, cells were exposed to an ultraviolet light source (Quarz Lampen GmbH, Hanau, Germany; radiant element with 33 cm length) mounted 55 cm above the surface of the plate, for different periods of time. Plates were incubated at 28°C for up to 6 days in the dark, and colonies were counted. The experiment was repeated at least 3 times, with similar results. Tolerance to oxidative stress was tested by incubation of yeast with hydrogen peroxide (2 mM) in YPG medium at 28°C for 14 h, and optical density was measured every 2 hours, according to Park *et al*. (2000).

2.6 *GC-MS analysis*

Identification of acid metabolites secreted by yeast strains (1278b and its IM^R mutant) was performed with gas chromatography – mass spectrometry (GC-MS) as described before (Machnicka *et al.* 2004). Yeast cells were grown for 4 days at 30°C, with shaking, in a minimal medium. After centrifugation, 1 mL of the supernatant was lyophilized and dried for 2 days over P_2O_5. The residue was dissolved in 1 mL of n-butanol and acetyl chloride (10:1, v/v) mixture, derivatized at 100°C for 1 h and (evaporated under nitrogen). The residue was suspended in 600 μL ethyl acetate. The internal standard, inositol hexa-acetate, was added at 0.5 mg/mL. Aliquots (1 μL) were injected into a GC-MS apparatus using an automatic injection mode with a split ratio of 30:1. A bench-top HP GC-MS (HP5890/MSD5971) was used for GC-MS measurement. Separation was carried out on a HP1 glass capillary column (12 m × 0.2 mm). The oven temperature was increased by 15°C/min from 70 to 270°C with a final holding for 5 min. The temperature of the injection port was 250°C. Helium was used as a carrier, with a flow-rate of 50 mL/min. All spectra were analysed by the NIST MS Search Program. This test was repeated at least three times.

2.7 *Potassium leakage*

S. cerevisiae Σ1278b and EO25 mutant were cultivated at 28°C for 18 h in YPG medium. Cells were centrifuged, washed with PBS (phosphate buffer saline) and resuspended in glucose solution (50 mM glucose, pH 6.0) to obtain

optical density 0.6. IM was added at 80 μM concentration and after 30 min of incubation samples were centrifuged. Supernatants were analysed with atomic emission spectrometer (Varian AA240FS). Autoclaved and untreated cells were used, as positive and negative control, respectively.

2.8 *Plasma membrane isolation*

S. cerevisiae were cultivated with or without 80 μM IM at 28°C for 18 h in YPG. Cells were centrifuged, washed with distilled water and resuspended in TE buffer (10 mM Tris, 10 mM EDTA, pH 9.3) to obtain 10^9 cells/mL. 2-mercaptoethanol (Aldrich) was added (8 μL per 1 mL of cell suspension). Samples were incubated for 30 min at 37°C with shaking (100 rpm), centrifuged and washed with 1.2 M sorbitol. Cells were suspended in RT buffer (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM EDTA; pH 7.5). Zymolase (20 μg/mL) was added and cells were incubated for 1 h at 37°C. Obtained protoplasts were washed with 1.2 M sorbitol, suspended in distilled water and centrifuged (5 min, 5000 rpm, 4°C). Supernatants were ultracentrifuged for 1 h at 36 000 rpm (Beckman Coulter Optima L-90k). Plasma membranes were suspended in 1 mL of PBS.

2.9 *Ergosterol and fatty acid analysis*

Lipid phase was extracted from plasma membrane with chloroform:methanol according to Folch *et al.* (1957). Ergosterol content was measured from lipid films with gas chromatography and estimated from a standard curve.

For fatty acid measurements, samples 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 80°C for 1 h. Fatty acids were analysed by gas-liquid chromatography combined with mass spectrometry (GLC-MS) using a Hewlett-Packard 5971A system, as described before (Paściak *et al.* 2002).

2.10 *Protein isolation and immunochemistry analysis*

For protein isolation after long incubation with IM, the parental strain Σ1278b and its IM-resistant mutant EO25 were incubated in YPG medium with or without 80 μM IM to obtain an optical density of 0.8, and centrifuged.

For observation of changes in Pma1 level in the time of incubation with IM, both yeast strains were incubated to obtain OD = 0.6; then IM was added to the final concentration of 80 μM and cells were incubated for another 18 h. Samples were taken after 2, 4, 6, 10, 14 and 18 hours of incubation with IM. For immunoblotting, the proteins were extracted using the NaOH/β-mercaptoethanol/trichloroacetic acid method as previously described (Riezman *et al.* 1983). The total extract was solubilized in Laemmli buffer supplemented with 8 M urea and incubated at 37°C for 30 min. The total protein concentration was examined and optimized by SDS-PAGE electrophoresis followed by Coomassie staining. The equal amounts of TE proteins isolated from all samples were resolved in 10% SDS-PAGE and subjected to Western blotting analysis. The primary antibodies used to determine Pma1 were polyclonal rabbit anti-Pma1 1:10000 (a gift from M. Ghislain, University of Louvain; Louvain-La-Neuve) and monoclonal rabbit anti-Cdc28 1:2000 (GE Healthcare) was used as a loading control. The Page Ruler Prestained Protein Ladder (Fermentas) was used for the estimation of bands molecular weight. The test was repeated three times with similar results.

2.11 *RNA extraction and reverse transcription*

Total RNA was isolated from exponentially growing cells (in YPG medium) that were left untreated or exposed to 80 μM IM using the Total RNA Mini Kit (A&A Biotechnology). Subsequently 2 μg of RNA samples were treated with DNaseI (RNase-Free, Fermentas) following the manufacturer's instructions. The absence of genomic DNA was verified by PCR using specific primers for the *PMA1* gene: PMA1 RT-F (5′-TGTTCCGACAAAACCGG TACT-3′) and PMA1 RT-R (5′-ACATCAAGTCGTCTGGAGAA ACAC-3′). Reverse transcription was performed with 1.5 μg of purified RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA was verified by PCR using the primers for the *PMA1* gene.

2.12 *Quantitative PCR*

Real-time amplification was performed in the LightCycler® 480 Real-Time PCR System (Roche) with the Real-Time 2xPCRMaster Mix SYBR® kit (A&A Biotechnology) according to the manufacturer's instructions using 1 μL of cDNA and the primers: PMA1 RT-F and PMA1 RT-R, in a total volume of 15 μL. The following conditions of amplifications were applied: 1 min at 95°C; 45 cycles of 10 s at 95°C, 10 s at 55°C and 15 s at 72°C. The general quality assessment of the PCR results was based on the amplification and melting curve profile of the samples in relation to the assay controls (non-template controls). Melting curve analysis was performed to confirm the specific amplicons and to identify putative unspecific PCR products (e.g., primer dimers). Successive dilutions of two samples (0 time-point for Σ1278b and EO25) were used as a standard curve. All presented results were standardized using the housekeeping gene *IPP1*,

encoding for inorganic pyrophosphatase, with the following primers: IPP1F (5'-CTTTATTGGATGAAGGTGA-3') and IPP1R (5'-TTAATTGTTTCCAGGAGTC-3'). For each of the RNA extractions, measurements of gene expression were obtained in triplicate, and the mean of these values was used for further analysis. Relative quantification method was used to analyse the expression level of *PMA1* gene. Kruskal-Wallis test was performed to compare the *PMA1* expression level between the wild type and IM^R mutant (using Statistica 10).

3. Results

3.1 *Pleiotropic character of a mutation conferring resistance to IM*

In present work, we investigated whether the resistance mutation phenotype is specific to quaternary ammonium salts. The parental strain Σ1278b and its IM-resistant mutant EO25 were tested for their sensitivity to antifungal drugs, metals and other growth inhibitors. These results are shown in figure 2.

In our study, we observed that the IM-resistant mutant is resistant not only to QAS but also to azoles (clotrimazole, ketoconazole, fluconazole), cycloheximide (substrates for ABC transporters) as well as crystal violet (effluxed by MFS transporter). Additionally, this mutation is associated with a loss of sensitivity to DTT and metals (selenium, tin, arsenic), which disrupt redox homeostasis in the cell (Wysocki *et al.* 1997; Basso *et al.* 2008). Moreover, the IM-resistant mutant acquired resistance to nystatin, a common polyene antibiotic, which binds to the plasma membrane and disrupts its functioning (White *et al.* 1998).

Growth on a different carbon source was investigated, but no significant differences between the parental strain and the IM-resistant mutant were observed (data not shown). However, it was noted that mutant EO25 poorly metabolized non-fermentable carbon sources, like glycerol in comparison with the parental strain (figure 2).

To investigate whether quaternary ammonium salt IM impacts yeast growth in the presence of azoles, the IM-resistant mutant and its parental strain Σ1278b were incubated with IM, fluconazole or itraconazole, separately, as well as with a combination of IM and an azole compound. The results showed that in the presence of both IM and azoles (fluconazole or itraconazole), yeast viability dropped significantly (figure 3). The wild type showed about 10-fold decrease in CFU after treatment with itraconazole and IM used in combination in comparison to itraconazole and IM alone. Fluconazole strongly affected wild type's viability (CFU log reduction of about 3) however, in combination with IM it caused almost complete growth inhibition. EO25 mutant was resistant to azole drugs used alone (figure 3), as well as in combination with 50 μM IM (data not shown). Only the increase of IM concentration (100 μM) caused a significant reduction of CFU after incubation with combined azoles and IM (figure 3).

The tolerance of the parental strain and the IM-resistant mutant to ethanol, osmotic shock (NaCl), oxidative stress (H_2O_2) and UV was tested. These factors initiate environmental stress response gene expression in the cell (Panaretou and Piper 1990). It was noted in figure 4 that mutant EO25 exhibited resistance to ethanol, osmotic shock (NaCl) and oxidative stress (H_2O_2), as well as increased sensitivity to UV in comparison to the parental strain. Additionally, the sensitivity of the wild-type and IM^R mutant to DNA damaging agents was tested (hydroxyurea, methyl methanesulfonate, 4-nitroquinoline 1-oxide and 5-fluorouracil). There were no differences between the sensitivity level of the wild type and EO25 mutant (figure 5).

Moreover, it was shown that both tested strains secrete citric acid into the medium; however, mutant EO25 secreted twice as much as its parental strain. Additionally, the IM-resistant mutant secreted α-ketoglutaric acid, which was not observed for the parental strain (data not shown). The secretion of a large amount of α-ketoglutaric acid by IM^R mutant cells could suggest weak α-ketoglutarate dehydrogenase activity, resulting in the accumulation of this metabolite (Machnicka *et al.* 2004; Zhou *et al.* 2010).

3.2 *Potassium leakage*

To test whether quaternary ammonium salt IM influenced plasma membrane permeability of the wild-type yeast strain and its IM-resistant mutant, a potassium leakage after IM treatment was measured.

It was shown that the yeast mutant resistant to IM exhibits enhanced plasma membrane permeability towards potassium ions (about 3 times) in the physiological conditions, in comparison to the wild type. IM treatment caused almost 10- and 3-fold increase in potassium leakage from the wild type and the mutant cells, respectively (table 1).

3.3 *Plasma membrane lipid composition*

To investigate the influence of IM on plasma membrane lipid composition, the ergosterol and fatty acid contents in the wild type and IM-resistant mutant plasma membranes were analysed. It was shown that in the physiological conditions ergosterol level was not significantly changed in IM-

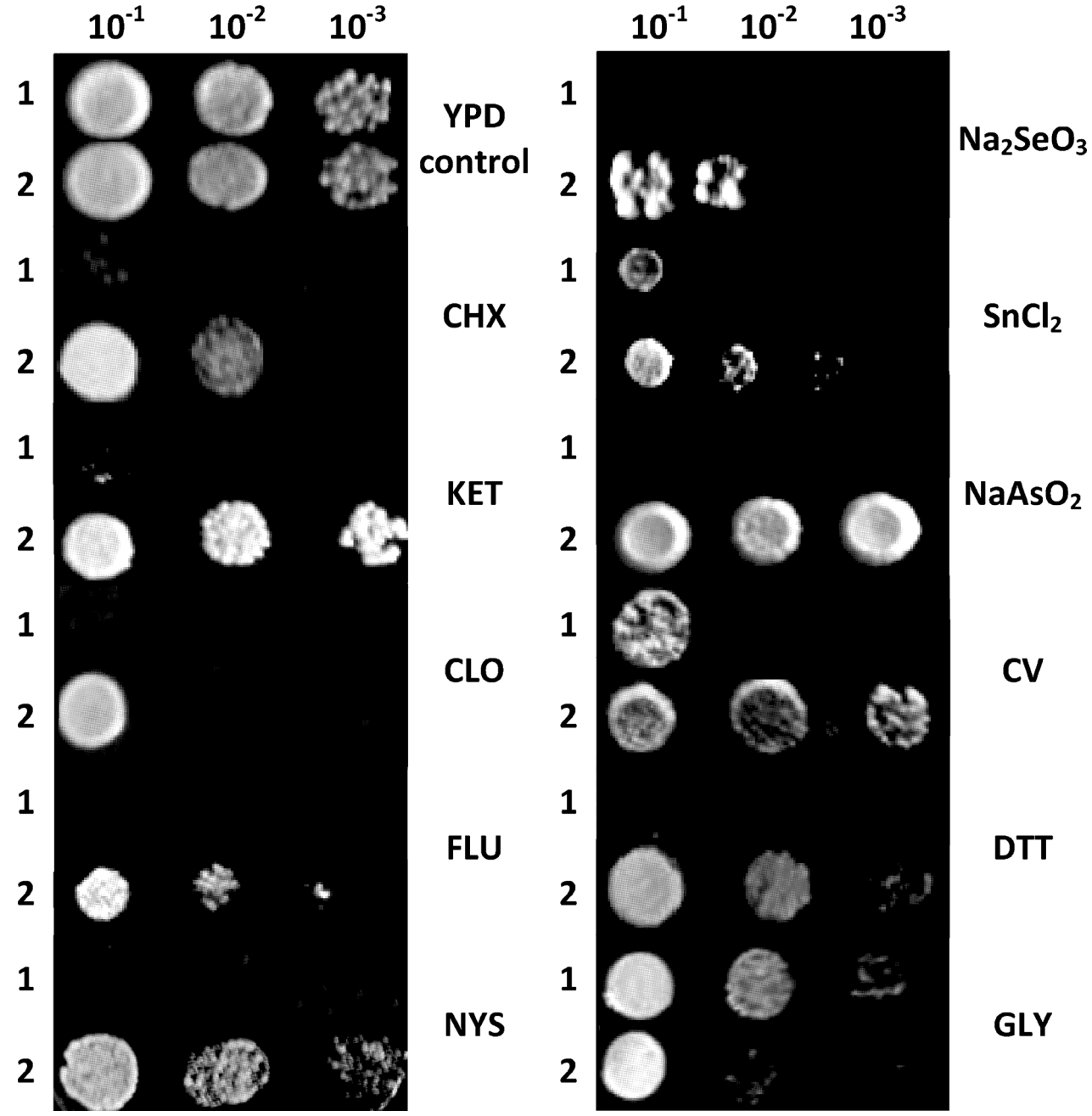

Figure 2. Sensitivity of the parental strain Σ1278b IM^S (1) and the IM-resistant mutant EO25(2) to various growth inhibitors. CHX, cycloheximide 90 ng/mL; KET, ketoconazole 10 μg/mL; CLO, clotrimazole 100 μM; FLU, fluconazole 0.3 mg/mL, NYS, nystatin 15 U/mg; Na_2SeO_3, sodium selenite 8 mM; $SnCl_2$, tin chloride 5 mM, $NaAsO_2$, sodium arsenite 0.75 mM; CV, crystal violet 30 μg/mL; DTT, dithiothreitol 20 mM, glycerol 2%. The culture with 1×10^8 cells/mL was diluted (10, 100 and 1000×) and 3 μL of yeast cells were spotted on solid YPG medium with the addition of given drug. The photographs were taken after 6 days of incubation. The phenotypic tests were repeated at least three times.

resistant mutant cells. After IM treatment the significant drop of ergosterol level both in the wild type (about 60%) and the mutant cells was observed however, the effect was much stronger in mutant resistant to IM (about 95%) (table 2).

The measurements of fatty acids showed that the wild type did not exhibit any significant differences in unsaturated fatty acids between control and IM-treatment conditions. The plasma membrane of mutant resistant to IM is slightly

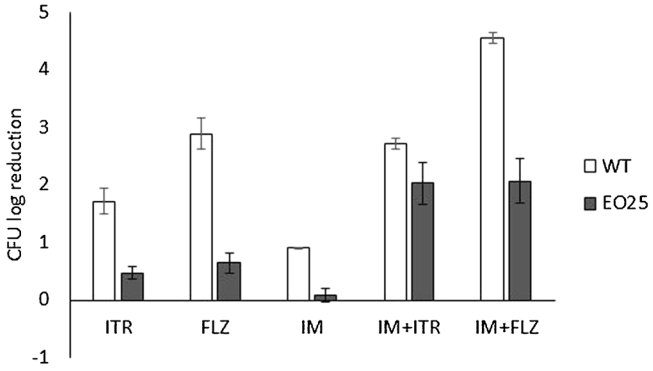

Figure 3. The reduction of colony-forming units (CFU) of the parental strain Σ1278b and the IM-resistant mutant EO25 after treatment with the IM (50 μM for 1278b or 100 μM for EO25), itraconazole (10 μg/mL), fluconazole (0.3 mg/mL) and in the combination of both compounds: IM and itraconazole or fluconazole. CFU log reduction was calculated from the ratio between CFU of untreated cells and cells treated with the compounds; mean ± SD, n=3.

more saturated than of the wild type's (in the physiological conditions), but the fatty acid analysis showed that the treatment of IM-resistant mutant with QAS caused increased unsaturation of the plasma membrane (table 3).

3.4 *PMA1 expression level*

The IM- resistant mutant showed similar phenotype to *pma1* mutants (resistance to ethanol, elevated temperature, osmotic shock and sensitivity to UV), what suggested that resistance to IM might be connected with the *PMA1* gene (Panaretou and Piper 1990). Thus, the *PMA1* gene of the parental strain and the IM-resistant mutant was sequenced; however, no differences between nucleotide sequences were detected (*PMA1* nucleotide sequence for Σ1278b strain was deposited in Gene Bank Database (NCBI, USA) HQ202157) (Altschul *et al.* 1997). Therefore, we decided to verify the Pma1 level of the parental strain and the IM-resistant mutant. Thus, Western blotting analysis was performed after incubation of both strains with or without IM. The results showed that the presence of IM caused a 6-fold increase in Pma1 level in the wild type and 8.5-fold increase in the IM-resistant mutant. Both strains grown without IM exhibited comparable levels of Pma1 (figure 6).

To investigate whether the Pma1 level changes during culture growth in the presence of IM, mid-log phase cultures (OD 0.6) of the parental strain and the IM^R mutant were exposed to IM, and Western blotting was performed after a given exposition time. The analysis showed that IM induced Pma1 production in both strains. However, in the IM^R mutant an increased Pma1 level was observed already after 2 h, whereas a comparable level of Pma1 in a parental strain was

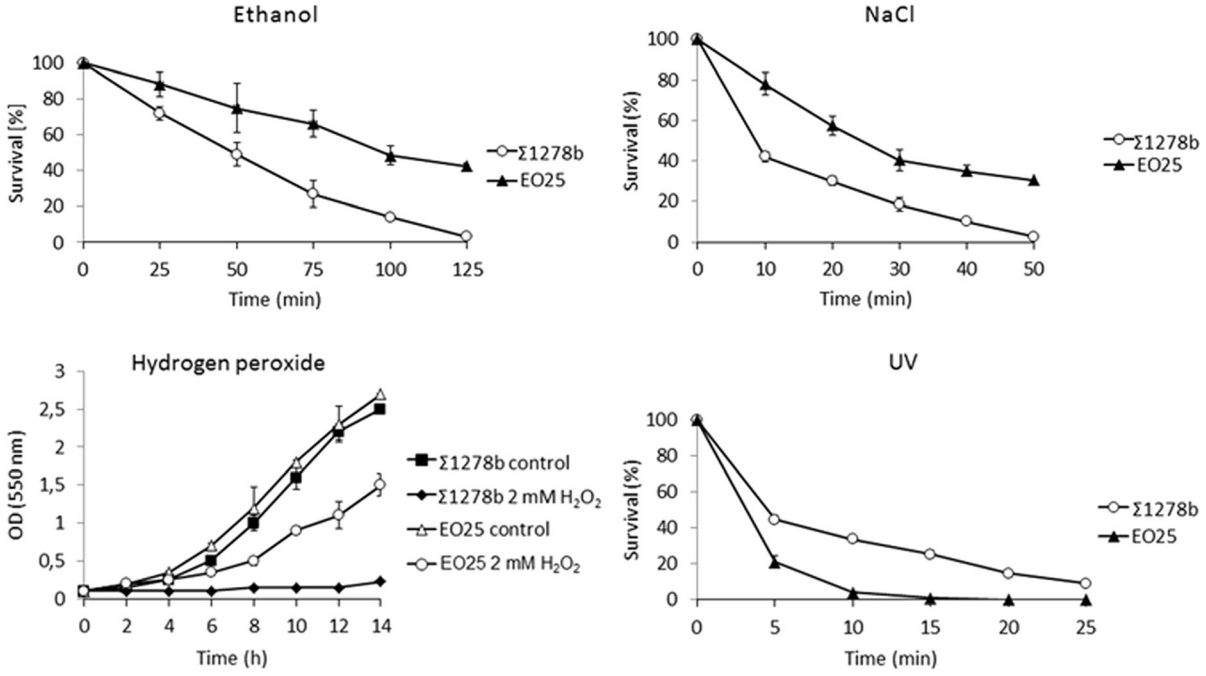

Figure 4. Sensitivity of yeast strains to: ethanol (12.5%); osmotic shock (2.5 mM NaCl); oxidative stress (2 mM H_2O_2); UV. The experiment conditions are given in Materials and Methods; mean ± SD; n=3.

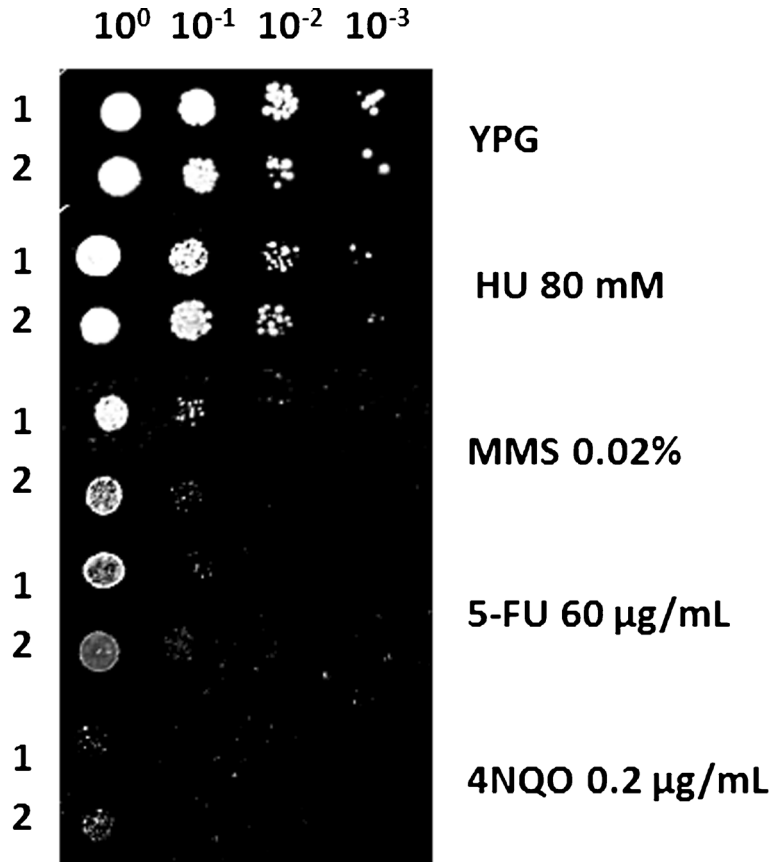

Figure 5. Sensitivity of the parental strain Σ1278b IMS (1) and the IM-resistant mutant EO25 (2) to DNA damaging agents: HU, hydroxyurea (80 mM); 5-FU, 5-fluorouracil (60 µg/mL); 4NQO, 4-nitroquinoline 1-oxide (0.2 µg/mL); MMS, methyl methanesulfonate (0.02%). The culture with 1×10^8 cells/mL was diluted (10, 100 and 1000×) and 3 µL of yeast cells were spotted on solid YPG medium with the addition of given compound. The photographs were taken after 6 days of incubation at 28°C.

reached only after ~6 h of incubation in the presence of IM (figure 7).

The induction of *PMA1* expression was determined by RT-PCR analysis, showing that the IM^R mutant exhibited a 2.5-fold increase in transcript level compared to the parental strain, after 6 h incubation with the compound. These observations are consistent with the increased level of Pma1 observed for the IM-resistant mutant. Further incubation showed a decrease in mRNA level in both strains (figure 8).

Table 1. The measurements of potassium ion leakage from yeast cells

Strain	Potassium leakage [ppm] from yeast cells after 30 min treatment with IM*
Σ1278b control	0.51 ± 0.016
Σ1278b + IM (80 µM)	5.83 ± 0.08
EO25 control	1.6±0.28
E025 + IM (80 µM)	5.53 ± 0.05

* Results represent mean ± SD of three independent experiments

4. Discussion

Quaternary ammonium salts (QASs) are commonly used in many fields of industry and medicine. Their bactericidal, fungicidal and antiviral activity has been demonstrated; however, the precise mechanism of QAS action is not yet fully understood (Obłąk and Krasowska 2010, Obłąk *et al.* 2013, 2014, 2015; Piecuch *et al.* 2016). The common application of QASs as disinfectants causes the acquisition of resistance, thus the synthesis of new compounds with stronger antimicrobial activity is needed (Hegstad *et al.* 2010).

The exploitation of resistant mutants is a universal tool for the investigation of mechanisms of drug action and cell resistance. In order to determine the genetic background of QAS resistance, the mutants resistant to quaternary ammonium salt (IM) were isolated (Obłąk *et al.* 1988). The IM^R

Table 2. Ergosterol level in plasma membrane of wild type and IM-resistant mutant after IM treatment

Strain	Ergosterol mass [mg per mg of plasma membrane lipids]*	The percentage content of ergosterol
Σ1278b control	0.04 ± 0.021	100
Σ1278b + IM (80 μM)	0.014 ± 0.003	35
EO25 control	0.034 ± 0.017	85
E025 + IM (80 μM)	0.002 ± 0.001	5

* Results represent mean ± SD of two independent experiments (each in three repetitions)

strain exhibited the pleiotropic phenotype. It was resistant to osmotic shock (sorbitol), antibiotics (chloramphenicol, erythromycin), ethidium bromide and DMSO. It also demonstrated tolerance to elevated temperature and was sensitive to SDS and $CaCl_2$ (Obłąk *et al.* 2010).

Currently, we showed that the IM^R mutant was also resistant to cycloheximide, crystal violet and azoles (ketoconazole, clotrimazole and fluconazole). The literature data indicate that these compounds are substrates for the ABC (Pdr5) and MFS (Sge1) transporters (Jacqout *et al.* 1997; Piecuch and Obłąk 2014). There is no information about the yeast genes directly involved in the resistance to QAS. Rogers *et al.* (2001) showed that the triple deletion of the *PDR5*+S*NQ2*+*YOR1* genes caused hypersensitivity of the yeast *S. cerevisiae* to tetradecyl trimethyl ammonium bromide, hexadecyl trimethyl ammonium bromide, and n-dodecyl trimethyl ammonium bromide. However, our results regarding IM resistance of mutants lacking *PDR1*, *PDR3* or *PDR5* genes did not show significant differences in the IM sensitivity level comparing to wild type (data not shown).

Quaternary ammonium salt IM enhanced the sensitivity of yeast cells to azoles. The active efflux of these drugs by PDR pumps is one of the most common mechanisms of azole resistance (Ferreira *et al.* 2005). To date, numerous substances reducing fungal azole resistance have been identified. These chemosensitizers can inhibit pump function by specific interactions, lowering intracellular ATP concentration or perturbations in the plasma membrane structure (Bartosiewicz and Krasowska 2009).

QASs are cationic detergents that exhibit high surface activity and strong reactivity towards biological membranes. They can affect the fluidity of the lipid bilayer, perturb specific hydrophobic and electrostatic interactions of membrane proteins and lipids, and change lipid asymmetry. QASs can inhibit membrane enzymes by rinsing the lipids (crucial for enzyme functions) out of the plasma membrane (Dufour and Goffeau 1980; Dubnickova *et al.* 2006). Quaternary ammonium salts, when applied at low concentrations, can change protein properties, while high concentrations solubilize proteins and lipids, leading to membrane destruction. Thus, QASs are commonly used as bactericides and fungicides (Obłąk and Krasowska 2010).

Plasma membrane is the first target for quaternary ammonium salts. We showed that both wild-type and mutant strain exhibit higher permeability of plasma membrane after exposition to QAS (potassium leakage). Furthermore, plasma membrane of mutant resistant to IM exhibit enhanced permeability in physiological conditions. Modification of biophysical properties of the plasma membrane is a common mechanism of microbial adaptation to rough environmental conditions (salt and oxidative stress, temperature and ethanol). Yeasts are able to change the amount of unsaturated phospholipids in order to maintain optimal liquid-crystal structure of the plasma membrane, necessary for generation of proton-motive force and nutrient transport (Dinh *et al.* 2008; de Freitas *et al.* 2012). Our research showed enhanced unsaturation of IM-resistant mutant plasma membrane after exposition to QAS. These changes are characteristic for ethanol and NaCl adaptation mechanisms (Lei *et al.* 2007; Turk *et al.* 2007). Mutant resistant to QAS exhibit higher tolerance to these factors in comparison to the wild type. The fluidization of the plasma membrane could be an adaptive response to quaternary ammonium salts.

Table 3. The plasma membrane fatty acid analysis in wild type and IM^R mutant cells after IM treatment

Strain	Total fatty acids (%)	Saturated fatty acids (%)	Unsaturated fatty acids (%)	Unsaturated/saturated fatty acids ratio
Σ1278b control	100	34.8	65.2	1.87
Σ1278b + IM (80 μM)	100	37.52	62.48	1.66
EO25 control	100	40.8	59.2	1.45
E025 + IM (80 μM)	100	29.6	70.4	2.38

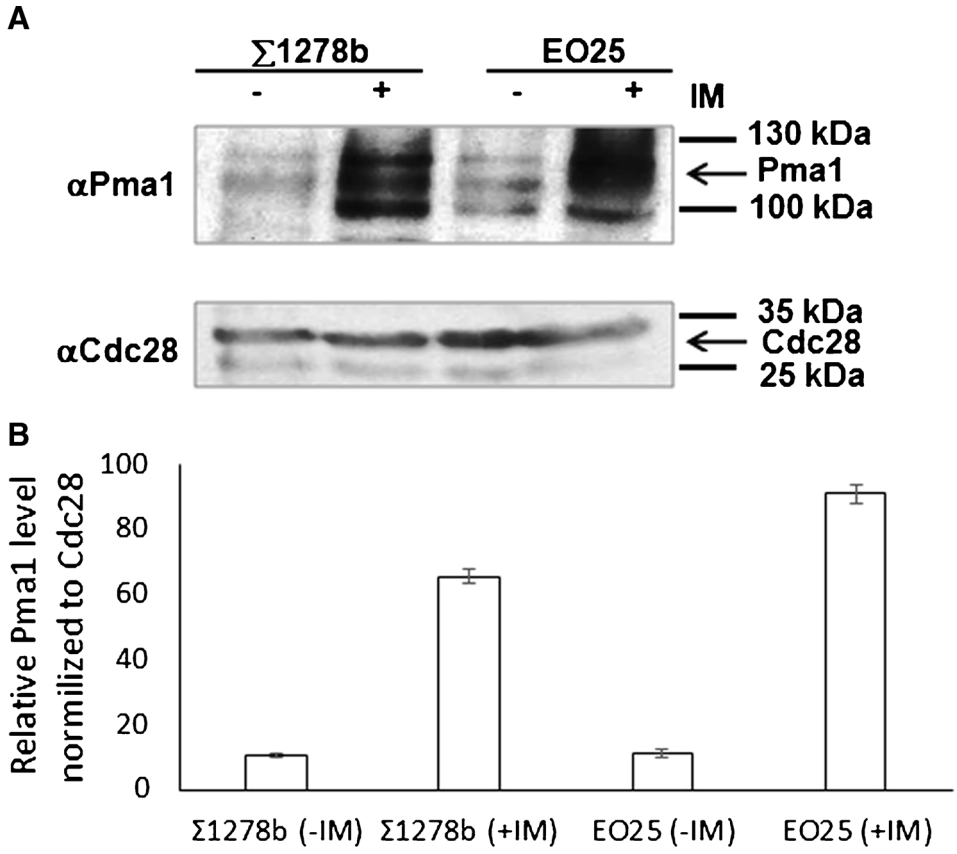

Figure 6. Pma1 level in cells of the parental strain (Σ1278b) and the IM-resistant mutant (EO25) detected by anti-Pma1 antibody. (−) cells untreated; (+) cells cultured in the presence of 80 μM IM: **A**, Western blotting of Pma1 and Cdc28; **B**, quantification of Pma1 level normalized to Cdc28 level as a loading control; mean ± SD, n=3. The optical density of the bands was quantified using Phosphoimager with ImageQuant software (Molecular Dynamics).

Plasma membrane lipid content plays a particular role in the resistance to various growth inhibitors. Numerous studies have shown that elevated ergosterol level in the plasma membrane is a common mechanism of resistance to some fungicides, e.g., azole compounds (Kontoyiannis *et al.* 1999). Their mechanism of action concerns the ergosterol biosynthesis pathway (they inhibit the Erg11 enzyme) (Ferreira *et al.* 2005). Other fungal antibiotics – polyenes (e.g., nystatin) embed the plasma membrane, creating a channel for cellular component leakage, which impairs the proton gradient (White *et al.* 1998). The IM^R mutant was resistant to azole compounds (ketoconazole, clotrimazole, and fluconazole) as well as to polyenes (nystatin). This might suggest that its resistance

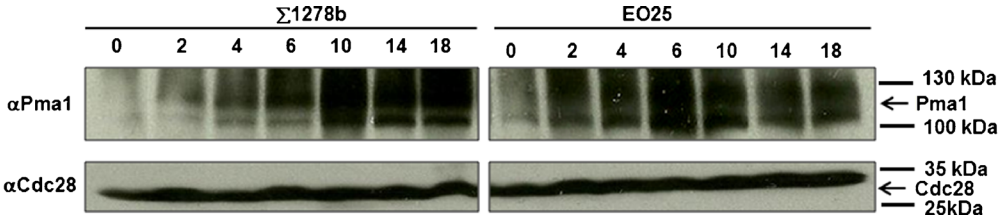

Figure 7. Western blotting analysis of Pma1 in physiological conditions (time 0) and in the presence of IM (80 μM) after 2, 4, 6, 10, 14 and 18 h for the parental strain Σ1278b and the IM-resistant mutant (EO25).

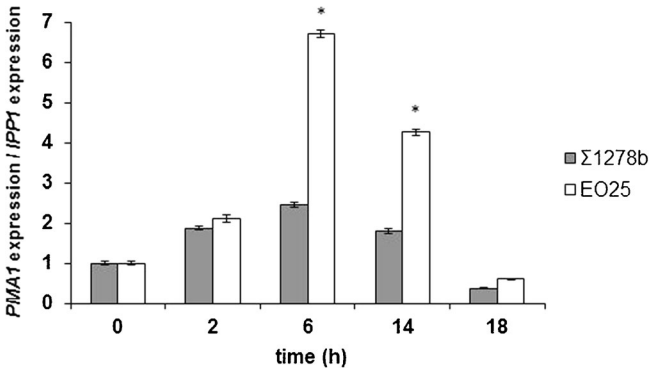

Figure 8. *PMA1* expression in the parental strain (Σ1278b) and the IM-resistant mutant (EO25) without IM (time 0) and after various times of exposition to IM (80 μM). *IPP1* encodes inorganic pyrophosphatase and is used as a reference gene; mean ± SD, n=3; *statistically different from the control ($p < 0.05$).

mechanism could be related to changes in the plasma membrane structure. On the other hand, the evaluation of ergosterol content in the plasma membrane of the wild type and IM^R mutant showed a drastic drop of ergosterol level after QAS treatment, especially in mutant cells. It is possible that IM might disturb biosynthesis of ergosterol and cause the accumulation of precursors in ergosterol biosynthesis pathway (e.g., squalene, lanosterol).

One of the most important plasma membrane enzymes is H^+-ATPase, responsible for maintenance of the plasma membrane electrochemical gradient, crucial e.g., for nutrient transport. Yeast H^+-ATPase (Pma1) is found in lipid rafts enriched in sphingolipids. Its activity strongly depends on plasma membrane lipid composition (Malinska *et al.* 2003; Grossman *et al.* 2007). The proper functioning of this enzyme requires negatively charged lipids (e.g., phosphatidyloinositol) as well as unsaturated fatty acids and sphingolipids (Rucka *et al.* 1983; Malinska *et al.* 2003).

Previously investigated pH sensitivity of the wild type and IM^R mutant showed that the mutant exhibited higher tolerance to low pH (4.5) (Obłąk *et al.* 2010). It might point at some alterations in the vacuole functioning, conferring tolerance to acidic environment. On the other hand, the medium was prepared with acetate buffer and the tolerance to weak organic acids is mediated by Pma1 activity (Ullah *et al.* 2012).

The IM^R mutant exhibited high temperature tolerance (48°C), resistance to osmotic stress (sorbitol, NaCl) and ethanol. IM^R mutant also showed sensitivity to UV however, it was not sensitive to other DNA damaging factors (hydroxyurea, methyl methanesulfonate, 4-nitroquinoline 1-oxide and 5-fluorouracil). The plasma membrane H^+-ATPase of the IM^R strain was resistant to quaternary ammonium salt IM *in vivo* and *in vitro* (Obłąk *et al.* 2000). A similar phenotype was observed for *pma1* mutants (Obłąk *et al.* 2010; Panaretou and Piper 1990), which could indicate the activity of the plasma membrane H^+-ATPase (encoded by *PMA1*), being responsible for IM resistance. Our results showed no differences in *PMA1* nucleotide sequence between the parental strain and the IM^R mutant. However, Western blotting revealed an increased Pma1 level after IM treatment in both strains, especially in mutant cells. The increased *PMA1* expression was observed in RT-PCR analysis; after 6 hours of exposition to IM the mutant exhibited a 2.5-fold increase in mRNA level in comparison to the parental strain. Pma1 is a crucial enzyme, a paradigm of a stable membrane protein with a half-life of >11 h (Benito *et al.* 1991). The activity of this protein is modulated mainly at post-translational level (by the phosphorylation of active sites). The increased expression of *PMA*1 after IM treatment might suggest upregulation of this gene by a transcription factor. There is not much data about transcription activation of Pma1 however, several putative binding sites for transcription regulators were found in the promoter region of *PMA1* and these include Gcr1 and Rap1 (Garcia-Arranz *et al.* 1994; Portillo 2000). Interestingly, single STRE element was found in *PMA1* promoter. This sequence is recognized and bound by Msn2 transcription factor, responsible for general stress response in yeast (Fernandes and Sa-Correia 2003). An IM-dependent raised Pma1 level suggests that this enzyme can play a role in cell detoxification from IM, but the question of whether it is a direct or indirect process needs further analysis.

QAS mechanisms of action can include: (1) direct inhibition of plasma membrane H^+-ATPase activity; (2) inhibition of proton transport; (3) alterations in the plasma membrane structure by embedding it or (4) lowering ATP level for H^+-ATPase.

The mechanism of resistance to quaternary ammonium salts in yeast is still poorly understood. Bacterial tolerance to these compounds is mediated by efflux pumps or by modifications in plasma membrane lipid composition and surface charge. Synthesis of altered LPS with decreased amount of negatively charged molecules reduces interactions with QAS (Dubnicková *et al.* 2006; Heredia *et al.* 2014). The obtained results point that QAS resistance in *Saccharomyces cerevisiae* could be connected with quantitative and qualitative alterations in the plasma membrane. Modifications in the lipid composition along with the elevated level of plasma membrane H^+-ATPase in the mutant cells and the faster

activation of *PMA1* transcription suggest that these factors play a role in QAS tolerance.

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