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Saccharomyces cerevisiae Mutants Affected in Vacuole Assembly or Vacuolar H^+-ATPase are Hypersensitive to Lead (Pb) Toxicity

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Abstract Lead is an important environmental pollutant. The role of vacuole, in Pb detoxification, was studied using a vacuolar protein sorting mutant strain (*vps16*Δ), belonging to class C mutants. Cells disrupted in *VPS16* gene, did not display a detectable vacuolar-like structure. Based on the loss of cell proliferation capacity, it was found that cells from *vps16*Δ mutant exhibited a hypersensitivity to Pb-induced toxicity, compared to wild type (WT) strain. The function of vacuolar H^+-ATPase (V-ATPase), in Pb detoxification, was evaluated using mutants with structurally normal vacuoles but defective in subunits of catalytic (*vma1*Δ or *vma2*Δ) or membrane domain (*vph1*Δ or *vma3*Δ) of V-ATPase. All mutants tested, lacking a functional V-ATPase, displayed an increased susceptibility to Pb, comparatively to cells from WT strain. Modification of vacuolar morphology, in Pb-exposed cells, was visualized using a Vma2p-GFP strain. The treatment of yeast cells with Pb originated the fusion of the medium size vacuolar lobes into one enlarged vacuole. In conclusion, it was found that vacuole plays an important role in the detoxification of Pb in *Saccharomyces cerevisiae*; in addition, a functional V-ATPase was required for Pb compartmentalization.

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Introduction

Lead (Pb) is one of the most threatening environmental contaminants due to its long persistence and toxicity. Pb is also one of the most common heavy metal contaminants in the environment. Anthropogenic sources are the main contributors for Pb pollution, which include: mining, smelting, industrial discharges, waste incineration and coal burning [1].

In *Saccharomyces cerevisiae*, Pb induces loss of proliferative capacity in the absence of alteration of membrane permeability [29]. Pb reduces the ratio DNA/RNA [5] and provokes DNA damage [31]. The presence of Pb causes a strong oxidative stress (intracellular accumulation of reactive oxygen species), which may be the trigger for cell death by apoptosis in *S. cerevisiae* [4].

Intracellular metals concentrations can be regulated by different mechanisms: efflux, chelation to metal-binding peptides and proteins (such as glutathione or metallothioneins) or compartmentalization [8]. The transport of metals from the cytosol into vacuoles or lysosomes is a common mechanism of detoxification in eukaryotic cells [30]. Cobalt, copper, nickel, and zinc are examples of metals sequestered in the vacuole of yeast cells [16, 21, 28, 30].

The vacuole is a dynamic structure; its morphology can change in response to different extracellular conditions [10, 15]. Yeast cells, in exponential phase of growth, in rich media, contain 2–5 medium size vacuoles. Vacuole coalescence into a single organelle occurs during stationary phase or upon transference of yeast cells to glucose starvation conditions. On the contrary, osmotic stress induces a rapid vacuolar fragmentation and volume reduction. The modification of vacuole number and size, in response to external stimuli, through the coalescence and fragmentation of membrane, corresponds to the uptake or release of water and ions from vacuole [15].

In *S. cerevisiae*, ~70 mutants were implicated in vacuolar protein sorting (*vps* mutants) [15]. The *vps* mutants have been classified into six classes (A–F) based on several criteria, including the vacuolar morphology of the mutants [2, 3, 22]. Class C *vps* mutants present extreme defects in vacuole biogenesis [2]; however, these mutants, display WT morphology of nuclei and mitochondria [2]. The four class C *VPS* genes, *VPS 11*(*PEP 5*), *VPS16*, *VPS18* (*PEP3*), and *VPS33,* code for proteins, which are essential for vacuole biogenesis [2, 3, 22].

Vacuolar H^+-ATPase (V-ATPase) is an ATP hydrolysis driven proton pump responsible for the vacuole acidification; V-ATPase regulates cytosolic pH and plays an important role in defence against oxidative stress provoked by hydrogen peroxide menadione and diamide [13, 15]. In yeast cells, V-ATPase is a large membrane-bound enzyme complex, constituted by 14 subunits arranged into two functional domains: a peripheral, called V_1, and a membrane associated, designated V_0 [13]. V_1 domain is constituted by eight different subunits (A–H subunits); ATP hydrolysis takes place at the interface of the A and B subunits [32]. V_0 domain is constituted by six subunits (*a*, *c*, *c′*, *c″*, *d*, and *e*), and is responsible for the translocation of protons through the membrane [13, 32].

The role of vacuole and V-ATPase in Pb detoxification has not been elucidated. In the present work, the susceptibility to Pb, evaluated by a clonogenic assay, of a strain deleted in *VPS16* gene, which resulted in the absence of a detectable vacuole, was compared with the respective WT strain. The involvement of V-ATPase in Pb detoxification, in the yeast *S. cerevisiae,* was studied using mutants lacking V-ATPase subunits (*vma* mutants). In addition, the changes in vacuolar morphology, in Pb-exposed cells, were investigated using the WT strain with the subunit B of the V-ATPase (Vma2p) fused with green fluorescent protein (GFP) (Vma2p-GFP strain).

Materials and Methods

Strains, Media, and Culture Conditions

Saccharomyces cerevisiae strains used in this work are listed in Table 1. Wild type (BY4741) and single gene deletion strains (*vps* and *vma* deletion mutants) were purchased from EUROSCARF collection (Frankfurt, Germany). Vma2p-GFP strain was obtained from yeast GFP clone collection (Invitrogen, USA).

The strains were routinely maintained at 4 °C on YPD agar slants [10 g/l yeast extract (Difco-BD), 20 g/l peptone (Difco-BD), 20 g/l glucose (Merck), and 20 g/l agar (Merck)]. Mutants were maintained under a selective pressure in YPD agar with 0.02 % (w/v) geneticin (Sigma-Aldrich).

Pre-cultures were prepared in 10 ml of YPD broth in 100 ml Erlenmeyer flasks. Cells were incubated at 25 °C on an orbital shaker, at 150 rpm, for 8–10 h. Cultures in exponential growth phase were obtained by inoculating 100 ml of YPD broth, in 250 ml Erlenmeyer flasks, with pre-cultures and grown overnight (OD_{600} ~1.0) under the same conditions as the pre-culture.

Treatment of Yeast Cells with Pb

After growth, cells were harvested by centrifugation (2,000 *g*, 5 min), washed twice with deionised water and resuspended in [2-(*N*-morpholino) ethanesulfonic acid] (MES) pH buffer (Sigma-Aldrich) 10 mmol/l, at pH 6.0, with 2 % (w/v) glucose, to ~1×10^7 cells/ml. MES is a suitable pH buffer for heavy metal toxicity studies because it does not complex Pb [27] and yeast cells maintain viability when incubated in this buffer for 48 h [26].

Cell suspensions (40 ml) containing 1×10^7 cells/ml, in 10 mmol/l MES buffer (pH 6.0), with 2 % (w/v) glucose and the appropriate volume of lead solution ($Pb(NO_3)_2$), from a stock standard solution of 2,000 mg/l (Merck) were shaken in 100-ml Erlenmeyer flasks at 150 rpm, at 25 °C.

Viability was determined by plating the cells on YPD agar. Thus, samples (1 ml; 2–3 replicates) were taken, serially diluted with sterile deionised water and plated (two replicates of 200 µl of the convenient dilutions). The colonies were counted after 3–4 days of incubation at 25 °C. The % of survivors was calculated using the number of colony-forming units (c.f.u)/ml at zero time as reference (100 %).

Fluorescence Microscopy

For vacuolar morphology analysis, cells from cultures in exponential growth phase (grown overnight to OD_{600} ~1.0) were analyzed by epifluorescence microscopy. Thus, 1×10^7 cells/ml were suspended in 10 mmol/l HEPES buffer (pH 7.4), containing 5 % (w/v) glucose and incubated with CellTracker Blue 7-amino-4-chloromethylcoumarin (CMAC; Invitrogen), in a final concentration of 100 µmol/l, for 30 min, at 25 °C, in the dark. CellTracker Blue CMAC stock solution of 10 mmol/l was prepared in dimethyl sulfoxide (Sigma-Aldrich) and stored at −20 °C. After staining, cells were washed and suspended in HEPES buffer with glucose. Cells were observed using an epifluorescence microscope equipped with a HBO 100 mercury lamp and the filter set A [excitation filter (band pass filter, BP) BP 340–380, dichromatic mirror 400 and suppression filter (long pass filter, LP) LP 425], from Leica. The images were acquired with a Leica DC 300F camera using 100× oil-immersion N plan objectives and processed using Leica IM 50-Image manager software.

Table 1 Yeast strains used in this study

Reference	Strain	Genotype	Comment
BY4741	Wild type (WT)	MAT*a*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0	Control strain
Y02783	*vps16*Δ	BY4741; *YPL045w*::*kanMX4*	Without any detectable vacuolar-like structure
Y03883	v*ma1*Δ	BY4741; *YDL185w*::*kanMX4*	Without subunit A of catalytic sector (V_1 domain) of V-ATPase
Y03266	v*ma2*Δ	BY4741; *YBR127c*::*kanMX4*	Without subunit B of catalytic sector (V_1 domain) of V-ATPase
Y00268	v*ma3*Δ	BY4741; *YEL027w*::*kanMX4*	Without subunit *c* (V_0 domain) of V-ATPase
Y07328	v*ph1*Δ	BY4741; *YOR270c*::*kanMX4*	Without subunit *a* (V_0 domain) of V-ATPase
95700 (YBR127c)	*V*ma2p-GFP	BY4741; *VMA2*-GFP	Subunit B of V_1 domain (Vma2p) of V-ATPase tagged at its C-terminus with green fluorescent protein (GFP)

Vacuolar morphology in Pb-exposed cells was analyzed using a Vma2p-GFP strain; this strain displays the B subunit of V_1 domain (Vma2p) of the V-ATPase tagged, at its C terminus, with GFP, which allows to localize the vacuolar membrane [24]. Cells from Vma2p-GFP strain in exponential growth phase (grown overnight to OD_{600} ~1.0) were washed and treated with Pb as the WT strain. Subsequently, cells were washed, suspended in HEPES buffer with glucose and observed using a epifluorescence microscope equipped with the GFP filter set (excitation filter BP 470/40, dichromatic mirror 500 and suppression filter BP 525/50), from Leica. The images were acquired and processed as described above.

Reproducibility of the Results and Statistical Analysis

The data reported are the mean ± SD, presented with 95 % confidence value of at least 4 independent experiments. Statistical differences between wild type (BY4741) and *vps* mutant strain were tested using unpaired *t* test. The means values of viability of wild type and mutants lacking V-ATPase subunits (*vma* mutants) were subject to one-way ANOVA followed by Tukey–Kramer multiple comparison method; the same statistical treatment was done among *vps* and *vma* mutant strains.

EC_{50} and EC_{90} values, which represent the concentration of the Pb that caused the loss of 50 and 90 % of cell population proliferation capacity (viability), respectively, compared to the positive control (cells not exposed to the Pb). EC values were calculated considering that the concentration–response relationship can be described by the probit function; EC values were obtained using weighted linear regression analysis on probit-transformed data (TOXCALC version 5.0.32, Tidepool Scientific Software).

Results and Discussion

Cells Lacking a Detectable Vacuolar-Like Structure Display a Hyper Sensitivity to Pb

Yeast vacuole is described as an organelle that is functionally analogous to mammalian lysosome and the vacuole of plant cells. Vacuoles are acidic compartments, playing an important role in autophagy, cytosolic pH and ions homeostasis and metabolites storage [6, 14, 18]. Another important role of vacuole is in the detoxification of heavy metals through its accumulation (compartmentalization) [30]. In the present work, the role of vacuole in Pb detoxification, in *S. cerevisiae* cells, was studied. For this purpose, the susceptibility to Pb of WT and a single gene deletion strain (*vps16*Δ) was compared; this mutant belongs to class C vacuolar protein sorting (*vps*) deletion strains. The lack of vacuole can be visualized using CellTracker Blue CMAC, which selectively stain the *lumen* of the yeast vacuole [9, 23]. Cells of WT strain, in exponential phase of growth, displayed typical vacuoles, which are comprised of multiple medium-sized fluorescent lobes (Fig. 1a). No vacuolar-like structure could be observed in cells of *vps16*Δ strain, stained with CellTracker Blue CMAC; in these cells, only a faint fluorescence was observed (Fig. 1a).

Since *vps16*Δ strain lacks any vacuolar-like structure, this strain is well suited to determine the impact of the absence of vacuole in Pb-induced toxicity. As it can be seen in Fig. 1b, the sensitivity of *vps16*Δ strain is very significantly different ($P < 0.001$) than the WT strain, to the action of Pb (for 100–500 µmol/l). Cells of the WT strain, incubated for 3 h, with 100 µmol/l Pb, displayed a viability (accessed by the ability to proliferate on YPD agar

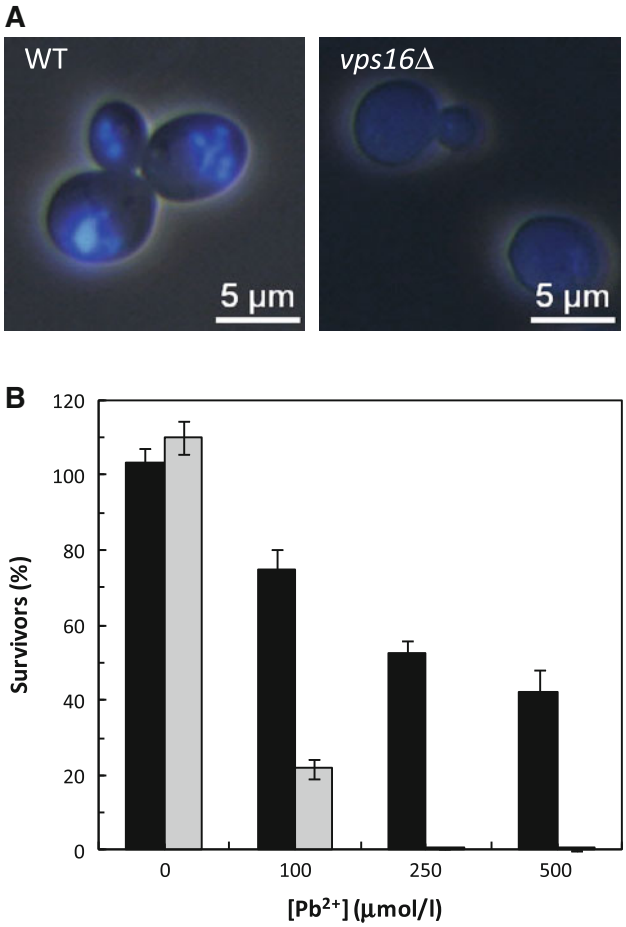

Fig. 1 Deletion of *VPS16* gene increase susceptibility to Pb-induced toxicity. **a** vacuole visualization using CellTracker *blue* CMAC stain. **b** viability of wild type strain (WT) BY4741 and *vps16*Δ mutant (strain without any vacuolar-like structure) exposed to Pb; 1×10^7 cells/ml, in exponential phase of growth, of the WT (*dark bar*) or the *vps16*Δ strain (*gray bar*) were suspended in 10 mmol/l MES pH buffer (pH 6.0), with 2 % (w/v) glucose, and treated with different Pb concentrations, for 3 h. Viability was estimated by c.f.u. counts. Each bar represents the mean of four independent experiments. Standard deviations are presented with 95 % confidence limits (*vertical error bars*). For all Pb concentrations tested, the difference between WT and *vps* mutant are very significant (unpaired *t* test; $P < 0.001$) (Color figure online)

plates) of 75 %, while cells of *vps16*Δ strain, incubated in same conditions, displayed a viability of ~22 % (Fig. 1b). Pb toxic effect was exacerbated for higher metal concentrations. Cells of *vps16*Δ strain incubated with 250 or 500 µmol/l Pb presented a viability <1 and 0.1 %, respectively, while, cells of WT strain, with vacuole, displayed a viability of ~53 and 43 %, respectively (Fig. 1b). The EC_{50} and EC_{90} values for WT strain were 4.1 and 10.8 times higher, respectively, than the corresponding values for *vps16*Δ strain (Table 2); these results clearly show the sensitivity of the cells without any vacuolar-like structure to Pb. The hyper susceptibility of *vps16*Δ strain to Pb strongly suggested the involvement of the vacuole in Pb detoxification.

Table 2 Effect of lead (Pb) on the survival of *S. cerevisiae*

Strain	EC (µmol/l)	
	50	90
Wild type	266	1,349
*vps16*Δ	65	125
v*ma1*Δ	86	327
v*ma2*Δ	80	275
v*ma3*Δ	106	562
v*ph1*Δ	139	536

EC effect concentration

EC_{50} and EC_{90} are the concentrations of Pb that induces the loss of viability of 50 or 90 % of cell population, respectively. 1×10^7 cells/ml were suspended in 10 mM MES pH buffer (pH 6.0), with 2 % (w/v) glucose, in the presence of different Pb concentrations, for 3 h; viability was assessed by plating cells in YPD agar. Values were obtained from four independent experiments ($n = 16$)

Pb Detoxification Require Functional Vacuolar H^+-ATPase (V-ATPase)

Next, the role of H^+-ATPase vacuolar (V-ATPase) on the Pb detoxification was investigated. Four V-ATPase subunit deletion mutants (*vma* mutants) were used: two without one of the subunits of the catalytic V_1 domain, more specifically, without subunit A (*vma1*Δ) or B (*vma2*Δ) and two mutants strains without one of the subunits of the membrane V_0 domain: subunit *a* (*vph1*Δ) or *c* (*vma3*Δ).

It has been described that the deletion of any V-ATPase subunit gene (except *VPH1* and *STV1*, which encode organelle-specific isoforms) abolishes typical vacuolar acidification and provokes the loss of V-ATPase activity (Vma^- phenotype); Vma^- phenotype is characterized by sensitivity to high extracellular pH and inability to grow on non-fermentable carbon sources [12, 13]. Here, it was shown that single deletion of genes encoding V-ATPase subunits originated a highly significant ($P < 0.01$) increase of susceptibility to Pb (for all Pb concentrations and mutants tested) comparatively to cells from WT strain. EC_{50} and EC_{90} values of WT strain were 1.9–3.3 and 2.4–4.9 higher, respectively, than *vma* deletion mutants (Table 2). For 100 µmol/l Pb, the deletion of *VPH1* gene affected less significantly ($P < 0.05$), the susceptibility to Pb toxic effect in comparison with the disruption of the other three individual *VMA* genes tested (Fig. 2). A similar observation was described regarding the sensitive of *vph*1Δ mutant to the herbicide 2,4-dichlorophenoxyacetic acid [7] or hydrogen peroxide [17]. For a higher Pb concentration (250 µmol/l), mutants disrupted in the subunit A or B of the catalytic domain, where ATP hydrolysis takes place, displayed a significant ($P < 0.05$) susceptibility to Pb than the mutants deleted in the subunit *a* or *c* of the membrane domain. In the case of the highest Pb concentration tested (500 µmol/l), *vma2*Δ mutant

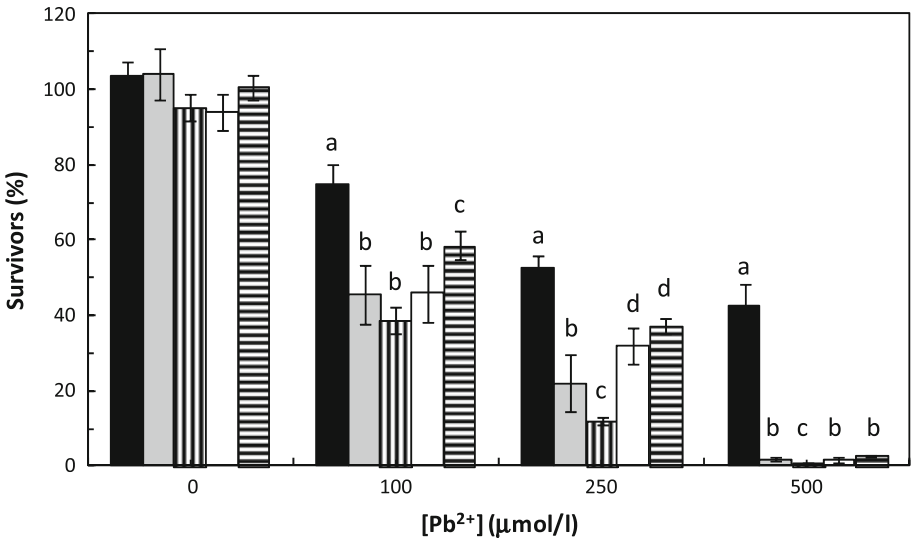

Fig. 2 Vacuolar H^+-ATPase subunits mutants (*vma* deletion strains) display an increased susceptibility to Pb. 1×10^7 cells/ml in exponential phase of growth of the WT strain (*dark bar*) or *vma1*Δ (*gray bar*), *vma2*Δ (*vertical line bar), vma3*Δ (*white bar*), and *vph1*Δ strains (*horizontal line bar*) were treated as described in Fig. 1. Viability was estimated by c.f.u. counts. Each bar represents the mean of four independent experiments. Standard deviations are presented with 95 % confidence limits (*vertical error bars*). Statistical differences among WT and *vma* deleted strains were subject to ANOVA followed by Tukey–Kramer multiple comparison method; for each Pb concentration, the means with *different letters* are significantly different ($P < 0.05$)

stain is significantly ($P < 0.05$) more sensitive than *vma1*Δ, *vma3*Δ, and *vph1*Δ strains (Fig. 2). It was described that *vma*2Δ and *vma3*Δ mutants are defective in V-ATPase activity, but possess morphological normal vacuoles [19]. The results obtained in the present work, with *vma* strains, indicate that the presence of a functional V-ATPase is important in defence against Pb toxic effect.

Mutant with defect in vacuole assembly (*vps16*Δ strain) is highly significant ($P < 0.01$) more sensitive to Pb toxic effect than the strains harboring an inactivated *VMA* gene (*vma* mutant strains). These results are in agreement with those reported by Szczypka et al. [28] about the role of vacuole in copper detoxification. In fact, *vps16*Δ strain corresponds to a more radical approach due to the absence of any vacuolar-like structure (Fig. 1a). Together, the results presented suggest the involvement of the vacuole in the sequestration of Pb, in *S. cerevisiae.* This detoxification pathway seems to require the presence of a functional V-ATPase.

In yeast cells, vacuole is an important calcium and iron storage compartment; vacuolar Ca^{2+}/H^+ exchanger, dependent of a V-ATPase-established H^+ gradient, seems to play an important role in calcium homeostasis [15]. Heavy metals are also sequestered in the yeast vacuole. The loss of V-ATPase activity compromises the activity of some transporters and the maintenance of vacuolar polyphosphates stores that can bind metal ions inside the vacuole [15, 16]. Similarly, Pb may be also sequestered in the vacuole, in a process dependent of H^+ gradient generated by the V-ATPase. This possibility is supported by the fact that *vma* mutants, which show a lack of a functional V-ATPase and a loss of vacuole acidification [12], also displayed an enhanced susceptibility to Pb-induced stress. In this context, the compartmentalization of Pb in vacuole can be seen as a form of regulation of Pb concentration in cytosol, in order to minimize toxic effects. A similar detoxification pathway has also been described in plant cells; once in the cytosol, the major part of Pb is sequestered in the vacuole, constituting a Pb detoxification mechanism [11, 20, 25].

Pb-Induced Vacuolar Morphology Modification

The morphology of yeast vacuole can change in response to different extracellular conditions [15]. The possible impact of Pb on yeast vacuolar morphology was observed using a strain expressing GFP-tagged *VMA2* (Vma2p-GFP strain), which allows localizing the vacuolar membrane. This method seems to be well suited for analyzing the impact of external stimuli on vacuolar morphology as it does not require the staining of vacuole membrane [10]. Cells collected in exponential phase of growth and incubated for 3 h, in MES buffer (pH 6.0) with 2 % (w/v) glucose, in the absence of Pb, retained its typical vacuolar morphology: the presence of multiple medium-sized lobes (Fig. 3). The exposition of the yeast cells to 500 µmol/l Pb, for 3 h, originated the fusion of the lobes into one enlarged vacuole. A transition between multiple medium-sized lobes to one enlarged vacuole was observed in the majority of the cells exposed to 250 µmol/l Pb (Fig. 3). The changing of vacuolar morphology from a fragmented structure comprising 3–4

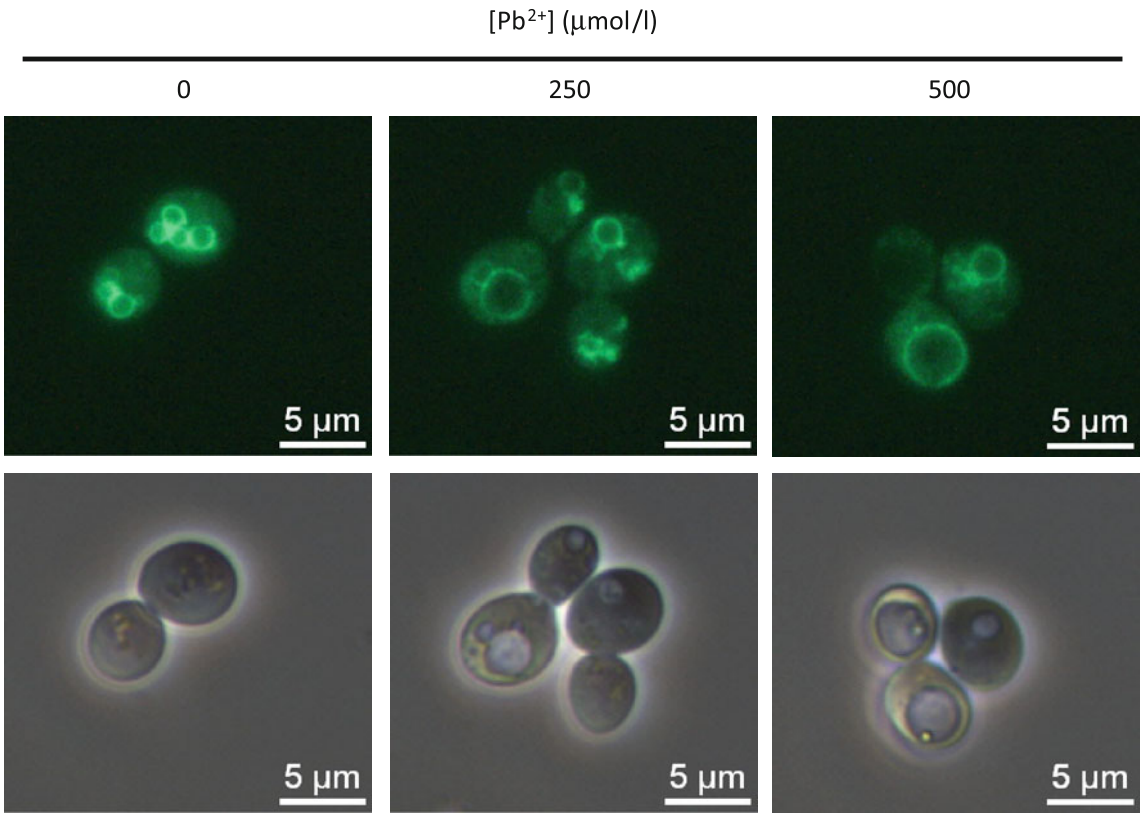

Fig. 3 Impact of Pb on vacuolar morphology. Vacuoles were visualized by observing the localization of Vma2p-GFP fusion protein. Cells from Vma2p-GFP strain, in exponential phase of growth, were treated as described in Fig. 1 and observed by fluorescence microscopy (*upper panel*) or by phase-contrast microscopy (*lower panel*)

medium size lobes to an enlarged vacuole could be the consequence of Pb accumulation in the vacuole.

In conclusion, it was shown that yeast cells harboring mutations in genes important either to vacuolar assembly (*VPS16*) or function (deletion of V-ATPase *VMA1*, *VMA2*, *VMA3* and *VPH1* genes), displayed an increased susceptibility to Pb toxicity. These data evidence the importance of vacuole in Pb detoxification in *S. cerevisiae*. Most likely, Pb is compartmentalized in the vacuole preventing its accumulation in cytosol and the resultant toxic effect; in this detoxification pathway, a functional vacuolar H^+-ATPase is required.

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