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ABCC Subfamily Vacuolar Transporters are Involved in Pb (Lead) Detoxification in *Saccharomyces cerevisiae*

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Abstract The present work has as objective to contribute for the elucidation of the mechanism associated with Pb detoxification, using the yeast *Saccharomyces cerevisiae* as a model organism. The deletion of *GTT1* or *GTT2* genes, coding for functional glutathione transferases (GST) enzymes in *S. cerevisiae*, caused an increased susceptibility to high Pb concentrations (500–1000 μmol L^{-1}). These results suggest that the formation of glutathione-Pb conjugate (GS-Pb), dependent of GSTs, is important in Pb detoxification. The involvement of ATP-binding cassette (ABC) vacuolar transporters, belonging to class C subfamily (ABCC) in vacuolar compartmentalization of Pb, was evaluated. For this purpose, mutant strains disrupted in *YCF1*, *VMR1*, *YBT1* or *BPT1* genes were used. All mutants tested, without vacuolar ABCC transporters, presented an increased sensitivity to 500–1000 μmol L^{-1} Pb comparative to wild-type strain. Taken together, the obtained results suggest that Pb detoxification, by vacuolar compartmentalization, can occur as a result of the concerted action of GSTs and vacuolar ABCC transporters. Pb is conjugated with glutathione, catalysed by glutathione transferases and followed to the transport of GS-Pb conjugate to the vacuole by ABCC transporters.

Keywords Glutathione transferases (GSTs) · Heavy metal · Lead (Pb) · Vacuolar ABC transporters · Yeast

Introduction

Lead (Pb) is one of the hazardous pollutants. Its presence in the environment has increased more than 1000-fold over the past three centuries as a consequence of human activity. Examples of environments that are Pb contaminated include mining areas, places near factories that produce, recycle or use lead, lead alloys or lead compounds, soil near roadway and older

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lead-painted houses [1]. In the last decades, several regulations were proposed in the European Union and USA in order to control the employ of Pb. As an example, the use of Pb was banned in gasoline additives, indoor paints, solders and water distribution systems. However, these sources of Pb can still persist. Renovation projects in older housing can produce airborne lead dusts from degraded paint. Drinking water in older houses or buildings containing lead pipes may contain lead [2].

Once inside the cells, heavy metals can display several toxicity mechanisms which include oxidative stress, DNA oxidation and enzyme inhibition [3]. The exposure of yeast cells of *Saccharomyces cerevisiae* to Pb induced a loss of cell proliferation capacity [4, 5], nuclear morphological alterations [6] and nuclear DNA damage [7–9]. In addition, Pb decreases the intracellular level of reduced glutathione [10] and induces the production of reactive oxygen species, which can be the trigger of programmed cell death by apoptosis [6].

Yeast cells can counteract metals' toxic effect through different detoxification processes. Examples of intracellular detoxification mechanisms include efflux, chelation and compartmentalization. In *S. cerevisiae*, metal removal from the cytosol through export pathways (efflux) was described for As(III) and Cd(II) through the export proteins Acr3p and Pca1p, respectively [11]. The reduction of free metal concentration in the cytosol can occur by metal chelation with low molecular weight, cysteine-rich peptides and proteins, such as glutathione (GSH) and metallothioneins [12]. Vacuolar sequestration of metals is a common detoxification mechanism in eukaryotes. It was described as the sequestration of Co, Cu, Ni and Zn in the vacuole of yeast cells [13]. Recently, it was also shown the involvement of the vacuole in Pb detoxification in yeast cells [14]. In *S. cerevisiae*, it has been reported that the ATP-binding cassette (ABC) transporter Ycf1p constitutes a major pathway for vacuolar compartmentalization of GSH-conjugated xenobiotics and metals, such as Cd and Hg [15–17]. Glutathione transferases 1 and 2 (Gtt1p and Gtt2p) participates in the detoxification process through the conjugation of the metals with GSH [18].

Members of ABC superfamily catalyse the ATP-dependent transport of a wide range of substrates across cellular membranes, such as plasma and vacuolar membrane. ABC transporters consist of two homologous halves that contain a membrane spanning domain (MSD) and a nucleotide-binding domain (NBD), which couples nucleotide hydrolysis to substrate transport [19]. Human ABC transporters have been divided into seven subfamilies (ABCA-ABCG) based on the relatedness of sequences within their NBDs [19]. ABC vacuolar transporters, belonging to class C (ABCC) subfamily, also called multidrug resistance-associated proteins (MRPs), is one of the best studied in yeast. Members of ABCC subfamily transport substrates that have first been conjugated with GSH [19]. The vacuole of the yeast *S. cerevisiae* harbours five ABCC transporters: yeast cadmium factor (Ycf1p) [16], vacuolar multidrug resistance (Vmr1p) [20], bilirubin pigment transporter (Bpt1p) [21], bile acids transporter (Ybt1p) [22, 23] and a less characterized transporter (Nft1p) [19, 24].

The yeast *S. cerevisiae* is a well-suited eukaryotic model organism for studying Pb toxic effects. Yeast cells share with animal and plant cells many similarities at organizational level. This yeast is widely used due to its cost effectiveness, rapid laboratory growth and easy genetic manipulation. Upon the conclusion of the yeast genome sequence project in 1996, *S. cerevisiae* became the first organism for which the complete list of ABC transporters was available [19]. Thanks to tools such as *S. cerevisiae* genome-wide deletion collection, further clues can be obtained about Pb detoxification pathways.

In the present work, the involvement of glutathione transferases (GSTs) on yeast tolerance to Pb was investigated. *S. cerevisiae* mutants lacking *GTT1* or *GTT2* genes, which codifies to

glutathione transferases 1 (Gtt1p) or 2 (Gtt2p), respectively, were used. In addition, the role of the ABCC subfamily transporters in the Pb detoxification was studied using mutants lacking the vacuolar transporters Ycf1p, Vmr1p, Ybt1p and Bpt1p. The importance of the vacuole in Pb detoxification was discussed.

Materials and Methods

Yeast Strains and Growth Conditions

S. cerevisiae strains used in this work are listed in Table 1. Wild-type (BY4741) and single-gene deletion strains were purchased from EUROSCARF collection (Frankfurt, Germany).

The strains were routinely maintained at 4 °C on yeast peptone dextrose (YPD) agar slants [10 g L^{-1} yeast extract (Difco-BD), 20 g L^{-1} peptone (Difco-BD), 20 g L^{-1} dextrose (Merck) and 20 g L^{-1} agar (Merck)].

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

Cells were harvested by centrifugation (2000×*g*, 5 min), washed twice with deionized water and resuspended in 10 mmol L^{-1} [2-(N-morpholino) ethanesulphonic acid] (MES) pH buffer (Sigma-Aldrich) at pH 6.0 with 2 % (*w*/*v*) glucose.

Cell suspensions (40 mL), containing 1×10^7 cells mL^{-1} in 10 mmol L^{-1} 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 2000 mg L^{-1} (Merck), were shaken in 100-mL Erlenmeyer flasks at 150 rpm at 25 °C for 3 h.

Table 1 *Saccharomyces cerevisiae* yeast strains used in this study

Reference	Strain	Genotype	Comment
BY4741	Wild type (WT)	MAT*a*; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0	Control strain
Y05973	*gtt1*Δ	BY4741; *YIR038c*::*kanMX4*	Without glutathione transferase 1 (Gtt1p)
Y01548	*gtt2*Δ	BY4741; *YLL060c*::*kanMX4*	Without glutathione transferase 2 (Gtt2p)
Y04069	*ycf1*Δ	BY4741; *YDR135c*::*kanMX4*	Without vacuolar membrane transporter Ycf1p
Y00928	*vmr1*Δ	BY4741; *YHL035c*::*kanMX4*	Without vacuolar membrane transporter Vmr1p
Y06425	*ybt1*Δ	BY4741; *YLL048c*::*kanMX4*	Without vacuolar membrane transporter Ybt1p
Y01503	*bpt1*Δ	BY4741; *YLL015w*::*kanMX4*	Without vacuolar membrane transporter Bpt1p

Cell Viability Determination

Yeast cell viability was assessed through a clonogenic assay consisting of removing samples (two to three replicates) of Pb-treated and non-treated cells, serially diluted with sterile deionized water and plated on YPD agar (two replicates of the convenient dilutions). The colonies were counted after 3–5 days of incubation at 25 °C. The % of viability was calculated using the number of colony-forming units (c.f.u) mL^{-1} at zero time as reference (100 %).

Cell Staining with Monochlorobimane

The staining of yeast cells with monochlorobimane (mBCl) was carried out as previously described [10]. Briefly, yeast cells were suspended at 1×10^7 cell mL^{-1} in 0.1 mol L^{-1} phosphate-buffered saline solution (PBS buffer) at pH 7.0 and incubated with mBCl (Sigma-Aldrich) in a final concentration of 100 μmol L^{-1} for 60 min at 25 °C in the dark. Cells were examined using a Leica DLMB 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 N plan objectives and processed using Leica IM50 Image Manager software.

Reproducibility of the Results and Statistical Analysis

All experiments were repeated, independently, three to six times. The data reported are the mean±SD, presented with 95 % confidence limits; the mean values were subject to unpaired *t* test.

Results

Glutathione Transferases are Involved in Pb Detoxification

Glutathione transferases (GSTs) are a group of enzymes that play an important role in detoxification of xenobiotics. In the yeast *S. cerevisiae*, it was identified and characterized two genes, *GTT1* and *GTT2*, codifying for functional glutathione transferase 1 (Gtt1p) and 2 (Gtt2p), respectively [18]. GSTs are important in the forming of conjugates of glutathione (GSH) in the cytosol. Their catalytic activity is associated with the ability to bind GSH and promote the formation of the thiolate anion (GS^-); once GS^- is formed, it is able of reacting spontaneously with electrophilic xenobiotics [18].

The involvement of glutathione transferases in Pb detoxification was evaluated by comparing the susceptibility to Pb of the wild-type (WT) strain with its isogenic mutants lacking the genes *GTT1* or *GTT2*. If Gtt1p or Gtt2p participate in Pb detoxification, mutant strains deleted in the gene *GTT1* (*gtt1*Δ strain) or *GTT2* (*gtt2*Δ strain) would be expected to be more sensitive to Pb than the WT strain. The survival, in the presence of Pb of the WT and *gtt1*Δ or *gtt2*Δ strains, was compared using a clonogenic assay in YPD plates. The strains *gtt1*Δ and *gtt2*Δ did not display an extremely significant ($P<0.001$) increase of susceptibility comparative to WT strain to 100 or 250 μmol L^{-1} Pb (Fig. 1). However, when exposed to high Pb concentrations (500 or 1000 μmol L^{-1}), the strains lacking Gtt1p or Gtt2p were extremely significant ($P<0.001$) more susceptible to Pb than the WT strain. After exposure for 3 h to 500 μmol L^{-1} Pb, *gtt1*Δ and *gtt2*Δ strains exhibited a viability of 6±1 % and 12±5 %,

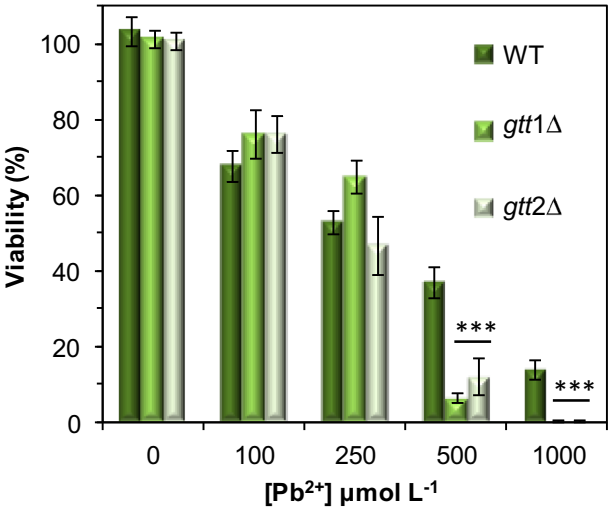

Fig. 1 Influence of glutathione transferases deficiency in the susceptibility of the yeast *S. cerevisiae* to Pb. Viability of wild-type strain (WT) BY4741 and the isogenic strains *gtt1*Δ and *gtt2*Δ without the gene *GTT1* and *GTT2* coding to glutathione transferases 1 and 2, respectively. 1×10^7 cells mL^{-1} were suspended in 10 mmol L^{-1} 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 three independent experiments. Standard deviations are presented with 95 % confidence limits (*vertical error bars*). The difference between the WT strain and each of the isogenic mutant strains was tested using an unpaired *t* test. The means with *asterisks* are extremely of significant difference ($P<0.001$)

respectively. In the same conditions, the viability of WT was 37±4 %. For the highest Pb concentration tested (1000 µmol L^{-1} Pb), the viability fell to 0.3±0.1 % and 0.6±0.3 % in *gtt1*Δ and *gtt2*Δ strains, respectively, whereas the viability of WT strain fell to 14±3 %. The increased susceptibility of *gtt1*Δ and *gtt2*Δ strains comparative to WT strain, to high Pb concentrations, strongly suggest the involvement of glutathione transferases in Pb detoxification.

ABCC Subfamily Mutants Display an Increased Sensitivity to Pb

Yeast cells are able to eliminate metals from the cytosol by GS-*X* pumps after their reaction with the thiol group of glutathione [15]. The results reported above, regarding the involvement of glutathione transferases, prompted us to test the susceptibility to Pb of the isogenic strains lacking ABCC subfamily vacuolar membrane transporters (*ycf1*Δ, *vmr1*Δ, *ybt1*Δ and *bpt1*Δ).

Ycf1p is the best characterized member of ABCC subfamily and presents the capacity to transport a broad range of xenobiotics substrates [19]. Monochlorobimane (mBCl) seems to be a useful substrate to access Ycf1p function. Monochlorobimane is essentially a non-fluorescent cell permeant probe. Once inside the cell, mBCl reacts with reduced gluthatione (GSH) to form glutathione-bimane (GS-B) conjugate, in a reaction catalysed by GSTs [25]. The GS-B conjugate has a blue fluorescence (emission maxima ~490 nm) [26]. In WT strain, GS-B conjugate was concentrated in the vacuole due to the presence of Ycf1p transporter. In these cells, GS-B conjugate could be observed, by microscopy, in the form of fluorescent-blue spot localized in the vacuole (Fig. 2a). In the cells of *ycf1*Δ mutant strain, GS-B conjugate was excluded from the vacuole being only possible to observe a faint fluorescence of the cytosol (Fig. 2a).

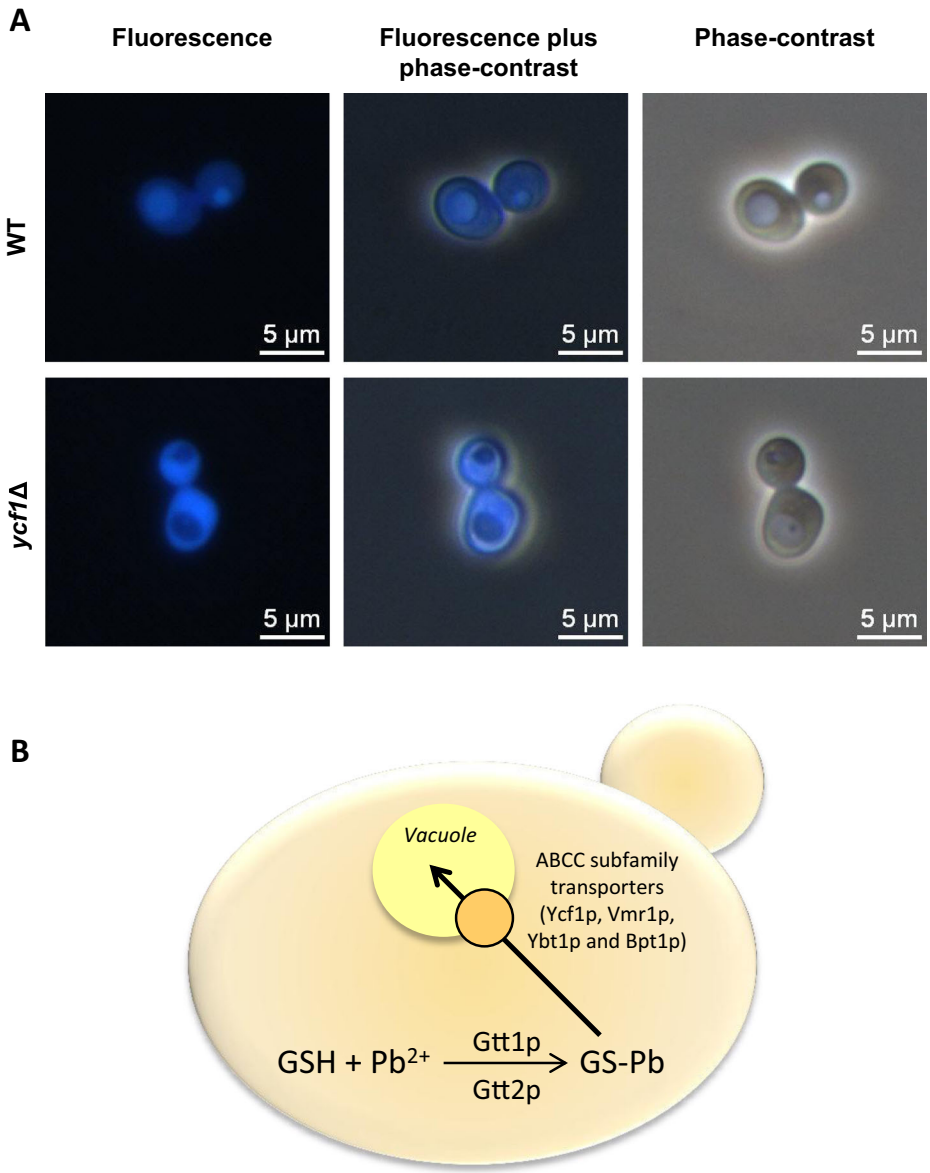

Fig. 2 Vacuolar compartmentalization of glutathione conjugates in *S. cerevisiae*. **a** Visualization of vacuolar sequestration of glutathione-bimane conjugate (GS-B). Cells of WT strain and the isogenic strain *ycf1*Δ (without the vacuolar ABCC transporter Ycf1p) were incubated with 100 μmol L^{-1} monochlorobimane for 60 min at 25 °C in the dark. A *blue* fluorescence associated with vacuolar accumulation of GS-B can be observed in WT strain. The vacuolar fluorescence was absent in *ycf1*Δ strain. **b** Schematic representation of the possible mechanism of Pb vacuolar sequestration mediated by ABCC transporters. Once inside the yeast cell, Pb ions are conjugated with glutathione (GSH). The formation of glutathione-Pb (GS-Pb) conjugate is mediated by glutathione transferases 1 (Gtt1p) and 2 (Gtt2p). Subsequently, GS-Pb is compartmentalized in vacuole through the action of ABCC vacuolar transporters (Ycf1p, Vmr1p; Ybt1p or Bpt1p)

A similar mechanism can be associated with Pb detoxification in yeast cells. In this scenario, vacuolar sequestration of Pb should occur in a two-step process: first, Pb should be conjugated with GSH, by the action of glutathione transferases; subsequently, GS-Pb should be transported to the vacuole by ABCC subfamily transporters (Fig. 2b). As it can be seen in Fig. 3, for low Pb concentrations (50–250 μmol L^{-1}), the ABCC subfamily mutants did not present an extremely significant ($P<0.001$) increase of susceptibility. The exposure to higher Pb concentration (500–1000 μmol L^{-1}) had, as consequence, an extremely significant ($P<0.001$) loss of viability in all ABCC mutants tested (Fig. 3). These results strongly suggest the involvement of the ABCC subfamily vacuolar transporters in the Pb detoxification.

Discussion

Cytosolic conjugation of organic xenobiotics with GSH (glutathione S-conjugation) is a common detoxification mechanism carried out by different types of cells [27]. In a previous work, it was shown that the exposure of *S. cerevisiae* cells to Pb originated a decrease of GSH [10], which suggested the participation of GSH in Pb detoxification. The results here presented support the involvement of glutathione transferases in Pb detoxification. In fact, mutants devoid in the genes *GTT1* or *GTT2* were more sensitive to the toxic action of high Pb concentrations comparative to the WT strain (Fig. 1). It suggest that Pb react, via glutathione transferases, with the thiol group of glutathione (GSH), resulting in the formation of a GS-Pb conjugates. A similar detoxification pathway was described for Cd [18, 28] and Hg [17].

The efflux of harmful compounds out of the cell as well as their transport and sequestration in the vacuole has the same benefit effect to yeast cells: removing hazardous chemical species from the cytosol and by this process, away from intracellular targets. In a previous work, it was shown the involvement of vacuole and the functional V-ATPase in Pb detoxification [14].

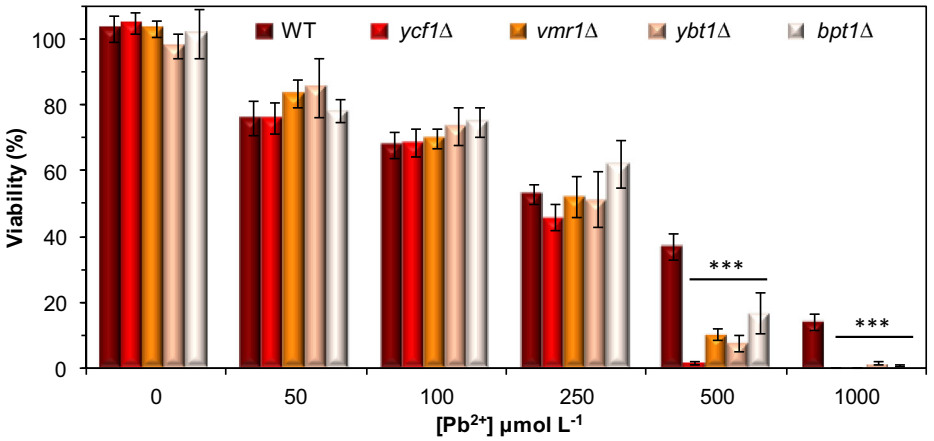

Fig. 3 *S. cerevisiae* strains deleted in ABCC subfamily transporters display an increased susceptibility to Pb of the wild-type (WT) strain. Isogenic strains lacking different ABCC subfamily membrane vacuolar transporters: yeast cadmium factor (*ycf1*Δ); vacuolar multidrug resistance (*vmr1*Δ), bile acids transporter (*ybt1*Δ) and bilirubin pigment transporter (*bpt1*Δ). The strains were exposed to Pb as described in Fig. 1. Viability was estimated by c.f.u. counts. Each bar represents the mean of 4–6 independent experiments. Standard deviations are presented with 95 % confidence limits (*vertical error bars*). Statistical differences between WT and each of the ABCC transporters deleted strains were tested using an unpaired *t* test. The means with *asterisks* are extremely of significant difference ($P<0.001$)

Taking into account that: (i) GSH is involved in Pb detoxification [10]; (ii) GSTs are involved in Pb detoxification (Fig. 1); (iii) ABCC proteins transport substrates as GSH conjugates rather than transporting the unmodified substrates themselves [19]; and (iv) cells of mutant strains devoid of the ABCC vacuolar transporters Ycf1p, Vmr1p, Ybt1p or Bpt1p displayed an increased susceptibility to Pb (Fig. 3), an alternative route of Pb transport to the vacuole emerges: Pb reacts with the thiol group of GSH in a reaction catalysed by GSTs, resulting in the formation of a GS-Pb conjugates which are then transported to the vacuole by ABCC subfamily transporters (Fig. 2b).

The function of ABCC subfamily proteins has been associated with the transport to the vacuole of a broad range of xenobiotics compounds, including heavy metals. *YCF1* gene has the ability to confer Cd resistance when overexpressed (hence, the name yeast cadmium factor) [16] and Cd hypersensitivity when deleted [25]. It was described the involvement of other members of the ABCC subfamily, namely Bpt1p [29] and Vmr1p [20], in Cd detoxification. In addition, it was suggested that Ycf1p can also transport other elements besides Cd, such as As(III), Hg(II) and Pb(II) [17, 30–32] evidenced by the inability of the y*cf1*Δ strain to grow in media containing these elements.

It should be pointed out that same care must be taken in the analysis of the results of such growth tests. Culture media can contain certain components, such as amino acids, that complex heavy metals, reducing their availability and toxicity. For instance, Prévéral et al. [32] reported that Pb was a little or no toxic to yeast cells. Probably, this low Pb cytotoxicity can be attributed to the exposure of yeast cells to metal in the solid culture medium. Similarly, a protective effect of media components was observed in Hg toxicity studies; probably, Hg was complexed by compounds of the rich medium [17]. In the present work, the toxic impact of Pb on yeast cells was evaluated in MES pH buffer. MES is a suitable pH buffer for heavy metal toxicity studies because it does not complex lead [33], and yeast cells remain viable [34]. Due to the absence of complexation, the total amount of Pb added was equal to the available metal present in solution. With this methodology, it is shown here for the first time, the involvement of ABCC subfamily vacuolar transporters in Pb detoxification.

Mercury is specifically transported into the vacuole by Ycf1p; Bpt1p and Ybt1p did not play a major role in this detoxification process [17]. On the contrary, Pb seems to be transported by all ABCC subfamily members tested: Ycf1p, Vmr1p, Ybt1p and Bpt1p. These results are in agreement with those which describe that Ycf1p exhibits an overlapping substrate specificity with its closest relative Bpt1p and other ABCC subfamily members [19].

Taking into account the accumulated knowledge obtained about Pb detoxification, a more global picture regarding the compartmentalization of Pb into vacuole can be obtained. Two parallel pathways for Pb sequestration can occur: one dependent of H^+ gradient generated by the V-ATPAse as previously described [14] and another mediated by ABCC transporters presented here. For Pb concentrations up to 250 μmol L^{-1}, the toxic effect was not felt in strains without glutathione transferase (Gtt1p and Gtt2p) or ABCC vacuolar transporters (Figs. 1 and 3). In these conditions, probably, yeast cells were able to sequester Pb in the vacuole, in a process dependent of H^+ gradient generated by the V-ATPAse. For Pb concentrations >250 μmol L^{-1}, the participation of vacuolar ABCC transporters seems to be important in the compartmentalization of Pb.

In conclusion, direct comparison between WT strain of *S. cerevisiae* and the isogenic strains harbouring single deletions of *YCF1*, *VMR1*, *YBT1* or *BPT1* genes demonstrated that these ABCC vacuolar transporters were required to increase the resistance to Pb. Most likely, Pb is conjugated with GSH in a reaction catalysed by glutathione transferases (Gtt1p and Gtt2p). Subsequently, GS-Pb conjugate is transported to the vacuole by the

action ABCC vacuolar transporters Ycf1p, Vmr1p, Ybt1p or Bpt1p. The vacuolar sequestration of Pb can help the cells to survive in the presence of this toxicant.

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