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Putative P_{1B}-type ATPase from the bacterium *Achromobacter xylosoxidans* A8 alters $Pb^{2+}/Zn^{2+}/Cd^{2+}$-resistance and accumulation in *Saccharomyces cerevisiae*

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ABSTRACT

PbtA, a putative P_{1B}-type ATPase from the Gram-negative soil bacterium *Achromobacter xylosoxidans* A8 responsible for $Pb^{2+}/Zn^{2+}/Cd^{2+}$-resistance in *Escherichia coli*, was heterologously expressed in *Saccharomyces cerevisiae*. When present in Zn^{2+}- and Pb^{2+}/Cd^{2+}-hypersensitive *S. cerevisiae* strains CM137 and DTY168, respectively, PbtA was able to restore Zn^{2+}- and Pb^{2+}-resistant phenotype. At the same time, the increase of Pb, Zn, and Cd accumulation in yeast was observed. However, Cd^{2+}-tolerance of the *pbtA*-bearing yeasts dramatically decreased. The PbtA-eGFP fusion protein was localized primarily in the tonoplast and also in the plasma membrane and the perinuclear region corresponding to the endoplasmic reticulum at later growth stages. This indicates that PbtA protein is successfully incorporated into membranes in yeasts. Since PbtA caused a substantial increase of Pb^{2+}/Zn^{2+}-resistance and accumulation in baker's yeast, we propose its further use for the genetic modification of suitable plant species in order to obtain an effective tool for the phytoremediation of sites polluted by toxic transition metals.

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1. Introduction

Transition metals, such as Zn, Cu, Fe, Mn, Mo, Ni, and Co, have an indispensable role in the metabolism of all living organisms [1]. However, when present in excess they, along with toxic non-essential metal ions, can pose a serious threat to living organisms. The mechanisms by which transition metals have a toxic effect are (i) production of reactive oxygen species and subsequent protein and lipid oxidation and DNA damage, (ii) inhibition of proteins through the binding to thiol groups of cysteines and nitrogen atoms of the histidine imidazolium cycle, (iii) interference with physiological metalome, and (iv) DNA mutagenesis or interference with DNA repair processes [2–5].

In order to prevent the occurrence of free transition metal ions in the cytoplasm, two main mechanisms have evolved to reduce the corresponding toxic effect. These include (i) the chelation of toxic metal ions with subsequent sequestration and (ii) metal transport from cytoplasm outside the cell or into the waste compartments (vacuoles) [6]. The efflux of transition metal ions from cytoplasm represents the main detoxification mechanism of divalent metal ions including Cd^{2+}, Pb^{2+}, and Zn^{2+} in bacteria [7]. The export of these ions from the cytoplasm is mediated by various transition metal transporters, mainly those from P-ATPase superfamily 1B-2 subgroup [8–10], Cation Diffusion Facilitator (CDF) family [11,12] and Resistance-Nodulation-Cell Division (RND) superfamily [7,13,14].

In addition to the efflux from cytoplasm, the detoxification of transition metal ions in yeasts is mediated by their chelation in complexes with Cys-rich compounds like glutathione (GSH), phytochelatins (PC), and metallothioneins. Free metal ions and their emerging complexes with GSH and PC can be sequestered in vacuoles through their transport across the tonoplast [15]. In *Saccharomyces cerevisiae*, a member of ATP-Binding Cassette (ABC) family Ycf1 transporter is responsible for vacuolar sequestration of glutathione S-conjugates of various transition metal ions including Cd^{2+} and Pb^{2+} [16,17]. Another *S. cerevisiae* ABC family member, Yor1, is localized in the plasma membrane and facilitates the efflux of glutathion-cadmium complexes in the same manner as Ycf1 [18]. Another protein in *S. cerevisiae* participating in detoxification of Cd^{2+} in cytoplasm is PCA1 from P_{1B}-ATPase superfamily. This protein transports Cd^{2+} ions across the plasma membrane outside the cell [19]. The function of Ycf1 and Yor1 in *S. cerevisiae* is replaced by two tonoplast-localized ABC-transporters Hmt1 and Abc2 in fission yeast *Schizosaccharomyces pombe*. These facilitate the efflux of Cd^{2+} in its complexes with phytochelatins from cytoplasm into the vacuole [20,21].

Cd and Pb represent unambiguously toxic transition metals without any beneficial effect in yeasts. Cells therefore aim to avoid the presence of Cd^{2+} and Pb^{2+} free form in the cytoplasmic space. Conversely, Zn is an indispensable micronutrient required for the proper function of many proteins. Therefore, the cell must possess mechanisms ensuring a sufficient pool of cytoplasmic Zn^{2+} ions and simultaneously avoid

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the presence of excessive amount of free zinc ions that could act detrimentally. Zinc homeostasis in eukaryotic cells is provided through its (i) active transport to the vacuoles, where it is stored in reusable form leading to diminishing of cytoplasmic zinc concentration, and (ii) active transport from the outside and remobilization from the vacuole in case of limited cytoplasmic zinc supplies (for review see [22]). In yeasts, the transport of excessive cytoplasmic zinc across the tonoplast to the vacuole is mediated through the action of two CDF proteins, Zrc1 and Cot1 [23]. ZIP family proteins, on the other hand, facilitate zinc uptake (Zrt1 and Zrt2) and remobilization (Zrt3) from the vacuole [23,24].

The information above is a short illustrative of the crucial role of transition metal protein transporters in Zn homeostasis and Cd/Pb detoxification in yeasts. In our previous work, several genes forming the *pbt* locus found on pA81 megaplasmid of the Gram-negative soil bacterium *Achromobacter xylosoxidans* A8 were characterized in the sense of their possible role in increased transition metal resistance of this bacterium [25]. A *pbtA* gene encoding for putative P_{1B}-ATPase was demonstrated to dramatically increase $Pb^{2+}/Cd^{2+}/Zn^{2+}$-resistance as well as decrease the accumulation of these metal ions in cells when heterologously expressed in $Pb^{2+}/Cd^{2+}/Zn^{2+}$-sensitive genotype of *Escherichia coli*. This study illustrates how a heterologous expression of PbtA, a putative bacterial P_{1B}-ATPase, influences the transition metal-associated phenotype of *S. cerevisiae*. We also researched subcellular localization of this putative transmembrane protein transporter in yeast cells.

2. Materials and methods

2.1. Yeast and bacterial strains

Yeast strains *S. cerevisiae* DTY168 (*MATα ycflA::hisG ura3-52, leu2-3,-112 his6* [16]) and *S. cerevisiae* CM137 (*MATα can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 zrc1::HIS3 cot1::URA3* [23]) were used for complementation tests. Metal accumulation assays and PbtA subcellular localization studies were performed using the strain *S. cerevisiae* W303 (*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* [26]).

Yeast cultures were grown at 30 °C. The medium "rich YPD" (20 g bacto-tryptone, 10 g yeast extract, and 20 g glucose per liter) or "minimal SD medium" (6.7 g yeast nitrogen base [Difco], and 20 g glucose per liter) were used for yeast cultivation, the latter supplemented with necessary auxotrophic nutrients if required. The Zn^{2+}/Cd^{2+} resistance assay was performed in SD medium. In case of Pb^{2+} testing, basic mineral medium was used (12.5 mM HEPES pH 7.0, 50 mM NaCl, 20 mM NH_4Cl, 1 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM $MnCl_2$) with the addition of 0.95 mM β-glycerolphosphate, 1 mM citrate, 2% glucose and 1 mg. l^{-1} uracil. Stock solutions of $CdCl_2$, $ZnCl_2$ or $Pb(NO_3)_2$ were filter-sterilized and added separately to achieve the desired concentration.

Growth of yeast suspension cultures was determined by optical density measurements at 600 nm (OD_{600}).

2.2. Expression vectors construction

In order to obtain vectors enabling expression of *pbtA* gene in yeasts, conventional cloning techniques were used. A *pbtA* coding sequence (AXYL_06634, positions 87173–90085) was amplified by PCR using primers 5′-ATGAACAAAGAATCCCCCAACAC and 5′-TCAGGCCTTCTGGG TCTTGAGTAG, and the PCR product was inserted into p416GPD centromeric "shuttle" vector [27], yielding the pGPD-pbtA plasmid.

Site directed mutagenesis of the *pbtA* coding sequence was performed according to the Stratagene Quikchange protocol. Briefly, A1994 in the GAC triplet encoding for phosphorylatable aspartate residue in the DKTGT motif was replaced by G leading to the glycine-encoding triplet GGC; primers used were 5′-GGCACTCGCATTGGG CAAGACCGGCACGC and 5′-GCGTGCCGGTCTTGCCCAATGCGAGTGCC (mutated position is underlined). The resultant altered *pbtA* coding sequence was reinserted into p416GDP leading to the pGPD-pbtA/A1994G vector.

For PbtA subcellular localization studies, a vector bearing *pbtA* and *egfp* (enhanced green fluorescent protein) fusion gene was constructed. The *pbtA* coding sequence in the p416GPD vector was fused with *egfp* (Clonetech) at its 3′-end. The resulting plasmid pGPD-pbtA-egfp carries *pbtA* and *egfp* coding sequences separated by the oligonucleotide 5′-GGT ACT AGT AGC encoding for GlyThrSerSer spacer in the reading frame.

A vector pGPD-pbtA/A1994G-egfp bearing mutated *pbtA* version fused with *egfp* at its 3′-end was prepared analogously to pGPD-pbtA-egfp.

Primary sequences of all prepared expression vectors were verified by DNA sequencing.

Yeast cells were transformed using the LiAc/ssDNA/PEG method and selected through *URA3* complementation [28].

2.3. Metal resistance assay

For the screening of Zn^{2+} and Cd^{2+} resistance, yeast cell cultures in mid-log phase were diluted to $OD_{600} = 1$, 10 μl droplets of diluted cell suspensions were put and spread onto the plates with SD medium containing $ZnCl_2$ or $CdCl_2$ in concentrations ranging from 100 μM to 500 μM or 1 to 50 μM, respectively. The growth was determined after 3 days of cultivation at 30 °C.

In order to determine the 50% inhibitory concentration (IC_{50}, the metal concentration that reduces the growth by half), the yeast cultures of initial $OD_{600} = 0.05$ were incubated in the presence of $ZnCl_2$, $CdCl_2$, or $Pb(NO_3)_2$ in concentrations 25 μM–0.8 mM, 1–50 μM, or 1 μM–1 mM, respectively. The cell growth was measured after 20 h of incubation and the data were adapted to the formula $OD(c) = OD(0) / [1 + \exp(c - IC_{50}) / b]$, where $OD(c)$ is the culture density at a given metal concentration c, $OD(0)$ is the density of the culture with no added metal and b is the slope of sigmoidal dose-dependent curve [12].

All metal resistance experiments were performed in duplicates and independently repeated three times.

2.4. Metal uptake assay

Yeast cells were harvested from the mid-log phase yeast culture ($OD_{600} = 1$) to obtain approximately 10^9 cells. The pellet was washed and resuspended in the 40 ml of 10 mM MES, 2% glucose (pH 6.1) spiked with 100 μM $ZnCl_2$, 10 μM $CdCl_2$ or 50 μM $Pb(NO_3)_2$, the latter supplemented with equimolar concentration of citric acid, and cultivated for 0.5 h at 30 °C. Cells were pelleted, washed twice by 10 mM MES, 5 mM EDTA (pH 6.1), and pelleted again. After air-drying, the cells were digested in 65% HNO_3 overnight under atmospheric pressure at ambient temperature. Metal content in diluted samples was measured by atomic absorption spectrometry (AAS; model Spectr AA300, Varian, Inc.). To express the relative contribution of *pbtA* gene to the metal uptake phenotype (U), the data were normalized using the formula $U = [(Me_{pbtA} - Me_{pGPD}) / Me_{pGPD}] \times 100$ (%), where $Me_{(pbtA)}$ is the metal content in yeast cells harboring *pbtA* gene and Me_{pGPD} is the metal content in cells transformed with the control vector p416GPD.

All metal uptake experiments were performed in triplicates and independently repeated three times.

2.5. Determination of PbtA subcellular localization

Either vector pGPD-pbtA-egfp or pGPD-pbtA/A1994G-egfp was introduced into *S. cerevisiae* W303 cells. For fluorescence analysis, the transformed cells were cultured in the SD medium. Yeast vacuolar membranes were stained by incubating cells with 0.032 μM FM4-64 fluorescent dye (Invitrogen, USA) for 20 min at 30 °C according to the recommendation of the supplier. Fluorescence signals corresponding

to eGFP-fusion protein and FM4-64 dye were monitored by fluorescence microscopy (Cell^R fluorescence microscope, Olympus).

3. Results

3.1. Heterologous expression of pbtA alters the phenotype of transition metal-sensitive S. cerevisiae strains

In order to reveal the effect of heterologous expression of *pbtA* gene on metal resistance phenotype of yeasts, complementation tests in *S. cerevisiae* transition metal-hypersensitive strains were performed. To accomplish this task, a vector based on the centromere plasmid p416GPD bearing *pbtA* coding sequence under the control of a constitutive GPD (glyceradehyde-3-phosphate dehydrogenase [27]) promoter was prepared and introduced in both *S. cerevisiae* strains CM137 (Zn^{2+}-hypersensitivity,[23]) and DTY168 (Cd^{2+}/Pb^{2+}-hypersensitivity [16]). Resistance of transformants to Zn^{2+}, Cd^{2+}, and Pb^{2+} was tested on both solid and liquid SD-medium, where IC_{50} values could be obtained. However, Pb^{2+}-resistance tests on a solid medium could not be performed because of either a very poor growth of yeast colonies or Pb^{2+} insoluble compound precipitation.

Expression of *pbtA* caused a significant increase in Zn^{2+}-resistance in the strain CM137 (Fig. 1, Table 1). The IC_{50} of CM137 carrying pGPD-pbtA rose from 148 ± 7 μM Zn^{2+} (IC_{50} of a negative control represented by yeasts bearing p416GPD without the insert) to 388 ± 14 μM (approximately 2.6-fold increase). When expressed in DTY168 strain, the gene *pbtA* also largely increased the Pb^{2+} resistance; IC_{50} increased from 61 ± 17 μM Pb^{2+} (control cells) up to 173 ± 35 μM (approximately 2.8-fold increase). To the contrary, DTY168 growth in the presence of Cd^{2+} was severely affected — IC_{50} dropped from 23 ± 1 μM Cd^{2+} for the control strain to the value lower than 0.5 μM (more than 40-fold decrease).

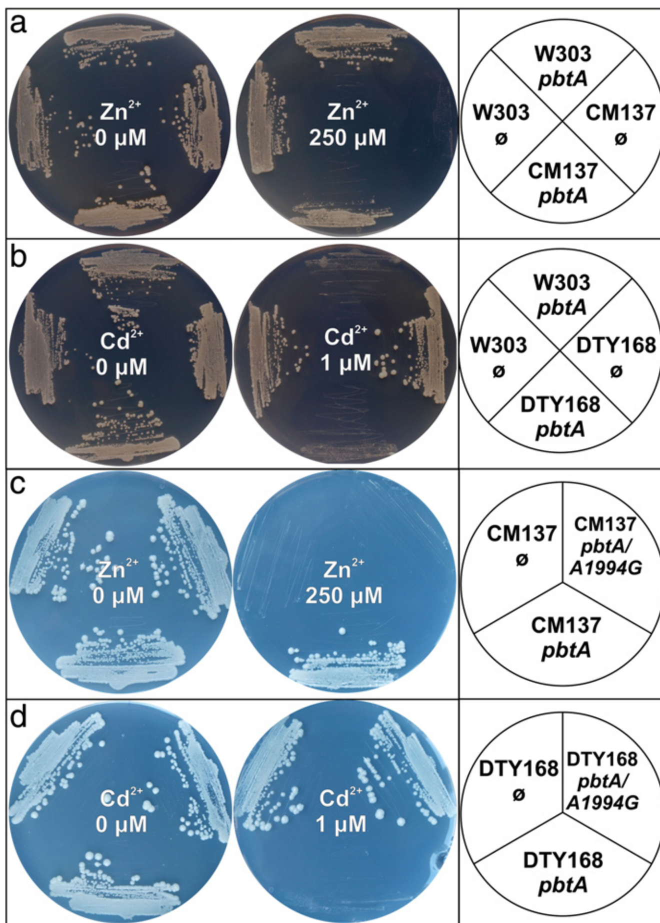

Fig. 1. Effect of Zn^{2+} and Cd^{2+} on yeast growth on solid medium. Yeast transformants bearing either pGPD-pbtA or pGPD-pbtA/A1994G or p416GPD without insert (negative control) were cultured to the mid-log phase in the selective SD medium. 10 μl droplets of these cultures were spread onto the solid SD medium containing appropriate concentration of $ZnCl_2$ (a, c) and $CdCl_2$ (b, d). The plates were cultured for 3 d at 30 °C. Note that *pbtA* rescued the growth of the CM137 Zn^{2+}-sensitive yeast mutant whereas it severely affected the growth of the DTY168 Cd^{2+}-sensitive strain as well as isogenic wild-type W303 even at very low Cd^{2+} concentration. The DTY168 and CM137 cells bearing mutated *pbtA* version exerted the same phenotype as negative control strains.

Table 1
Changes in metal resistance phenotype of *S. cerevisiae* Zn^{2+} and Pb^{2+}/Cd^{2+}-sensitive strains caused by *pbtA* expression.

S. cerevisiae strain[a]	Metal ion	$IC_{50\ Metal}$ value[b] [*b* value[c]] (μM)		
		p416GPD (control)	pGPD-pbtA	pGPD-pbtA/ A1994G
CM137	Zn^{2+}	148 ± 7 [43]	388 ± 14 [111]	141 ± 7 [32]
DTY168	Pb^{2+}	61 ± 17 [64]	173 ± 35 [54]	64 ± 9 [34]
	Cd^{2+}	23 ± 1 [6]	≤0.5[d]	23 ± 1 [4]

[a] No changes in Zn^{2+}- and Pb^{2+}-resistance phenotype were observed when *pbtA* was expressed in W303 strain. Contrarily, Cd^{2+}-resistance of the *pbtA*-bearing W303 cells decreased in the same manner as in the case of DTY168 strain.
[b] $IC_{50\ Metal}$, 50% inhibitory concentrations (metal ion concentrations required to decrease the optical density at 600 nm [OD_{600}] of 20-h culture to one-half of the positive control without any metal ion added). Values are given with standard deviations (three replicates). $IC_{50Cd/Zn}$ measurements were conducted in SD media and IC_{50Pb} in MJS media containing 1 mM citrate to prevent Pb^{2+} from precipitation.
[c] *b*, slope of the sigmoidal dose-dependent curve.
[d] 0.5 μM Cd^{2+} was the lowest concentration tested in IC_{50Cd} assay and it reduced the growth approximately by a half.

3.2. pbtA heterologous expression increases Pb, Zn and Cd accumulation in S. cerevisiae

In addition to the complementation studies, the impact of *pbtA* expression on metal uptake by yeast cells was also investigated. *S. cerevisiae* W303 cells carrying individual p416GPD (control strain) and pGPD-pbtA plasmid grown to the mid-log phase were exposed to 50 μM $Pb(NO_3)_2$, 10 μM $CdCl_2$, or 100 μM $ZnCl_2$ for 0.5 h. When *pbtA* was expressed, yeasts accumulated 10.0 ± 0.3 μg of Pb, 0.055 ± 0.002 μg of Cd, and 1.34 ± 0.04 μg of Zn per 10^8 of cells in comparison with 1.42 ± 0.03 μg of Pb, 0.041 ± 0.001 μg of Cd, and 1.16 ± 0.05 μg of Zn per 10^8 of control cells. This represents 609%, 33%, and 15% accumulation increase for Pb, Cd, and Zn, respectively, in cells bearing the *pbtA* gene versus in control cells (Fig. 2).

3.3. Phosphorylatable aspartate residue in DKTGT motif is essential for both metalloresistance and accumulation phenotypical effect of pbtA in S. cerevisiae

In order to reveal whether the changes of metalloresistance and accumulation phenotype of yeasts bearing the *pbtA* gene were the consequence of PbtA transport capacity and not only of metal binding capacity of the overexpressed non-functional polypeptide, its phosphorylatable aspartate residue was altered to glycine residue in a DKTGT motif essential for ATPase activity. A vector pGPD-pbtA/A1994G bearing the mutated *pbtA* version was used for the transformation of *S. cerevisiae* strains DTY168, CM137, and W303. Resultant yeast strains were subsequently subjected to the metalloresistance/accumulation assays. Presence of the plasmid pGPD-pbtA/A1994G changed neither the Zn^{2+}- nor the Cd^{2+}/Pb^{2+}-sensitive phenotype of CM137 and DTY168 strains, respectively (Fig. 1, Table 1). IC_{50} value for Zn^{2+} in CM137 strain (141 ± 7 μM) was comparable with that of the negative control strain bearing an empty vector p416GPD (148 ± 7 μM). The same situation occurred for the DTY168 strain where IC50 values were 23 ± 1 μM Cd^{2+} and 64 ± 9 μM Pb^{2+} versus 23 ± 1 μM Cd^{2+} and 61 ± 17 μM Pb^{2+} for the control strain.

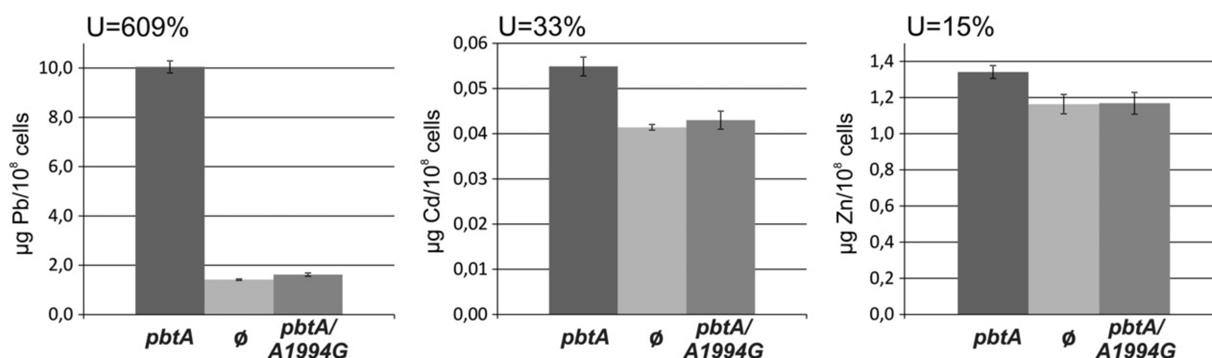

Fig. 2. Impact of *pbtA* and *pbtA/A1994G* expression on the Pb, Cd and Zn accumulation in *S. cerevisiae*. *S. cerevisiae* W303 cells carrying pGPD-pbtA or pGPD-pbtA/A1994G or plasmid p416GPD (control strain) grown to the mid-log phase were exposed to 50 μM Pb^{2+}, 10 μM Cd^{2+} or 100 μM Zn^{2+} for 0.5 h. The U value represents the relative contribution of the wild-type *pbtA* gene to the metal uptake in W303 cells.

When the phenotype of Pb/Zn/Cd-accumulation was tested, *S. cerevisiae* W303 bearing a *pbtA* mutated version accumulated 1.62 ± 0.07 μg of Pb, 0.043 ± 0.002 μg of Cd and 1.17 ± 0.06 μg of Zn per 10^8 of cells versus 1.42 ± 0.03 μg of Pb, 0.041 ± 0.001 μg of Cd and 1.16 ± 0.05 μg of Zn per 10^8 of control cells bearing p416GPD with no insert (Fig. 2). This represents 14% accumulation increase for Pb versus in control cells; changes in Cd/Zn-accumulation rate were statistically insignificant as tested by analysis of variance ($\alpha = 0.05$).

3.4. Subcellular localization of PbtA protein in yeasts

In order to reveal subcellular localization, the *pbtA* gene was fused at its 3′-end with the *egfp* coding sequence as a part of the p416GPD vector. The resultant fusion gene was expressed in *S. cerevisiae* W303 cells. According to the observations of eGFP and FM4-64 fluorescence signal co-localization, the PbtA-eGFP fusion protein was localized in the tonoplast (Fig. 3a–d). However, in later stage of the yeast growth, the PbtA-eGFP fluorescence signal of the fusion protein extended to the cytoplasmic membrane and into the perinuclear region (Fig. 3e–g).

To verify the efficient expression of the *pbtA* coding sequence bearing the A1994G mutation and the subcellular localization of the resultant PbtA/A1994G protein, p416GPD-based vector bearing the *pbtA/A1994G* gene fused at its 3′-end with the *egfp* was prepared and introduced into *S. cerevisiae* W303 cells. The observed localization pattern of the expressed PbtA/A1994G-eGFP fusion protein was similar to that of the wild-type PbtA-eGFP (Fig. 3h).

4. Discussion

This work was focused on how the phenotype of *S. cerevisiae* is changed after the heterologous expression of *pbtA* gene encoding for a putative P_{1B}-type ATPase from the bacterium *Achromobacter xylosoxidans* A8. A sequence of the PbtA polypeptide derived from the *pbtA* coding sequence consists of 970 amino acid (aa) residues. Using the TMHMM 2.0 tool [29], seven transmembrane spans (TM) were predicted in the PbtA protein sequence with two large 138 and 277 aa cytoplasmic loops between TM III–TM IV and TM V–TM VI, respectively. The DKTGT motif typical of all P-ATPases (in PbtA present in the form DKTGTLT) containing phosphorylatable aspartate residue is found starting at the position 639 [30]. Other conserved sequence motives common in P-ATPases were identified in the PbtA sequence, namely ATP-binding domain GDGXNDXP (in the form of GDGINDSP at the position 862) [31], PXXK (in the form of PEDK at the position 841) [32], and S/TGES (in the form of TGES at the position 517) [33].

The transmembrane metal binding XPX motif conserved in all P_{1B}-ATPases (in the form of CPC in the PbtA sequence; [34]) is found at the position 621. The HP locus involved in nucleotide binding is situated in the second large cytoplasmic loop at a distance of 38 aa behind the phosphorylated aspartate residue towards the carboxy-terminal [35]. Due to the presence of structural–functional signature motives $CPC(X)_4[S/T]XP$ (in the form of CPCALVISTP) and $N(X)_7K(X)_{20}DXH(X)_7N$ (in the form of NILVLALGIK$(X)_{20}$DMGASLLVVFN), PbtA can be classified into the 1B-2 subgroup of P-ATPases [34]. Members of this group are known to be involved in the $Zn^{2+}/Cd^{2+}/Pb^{2+}$-transport including

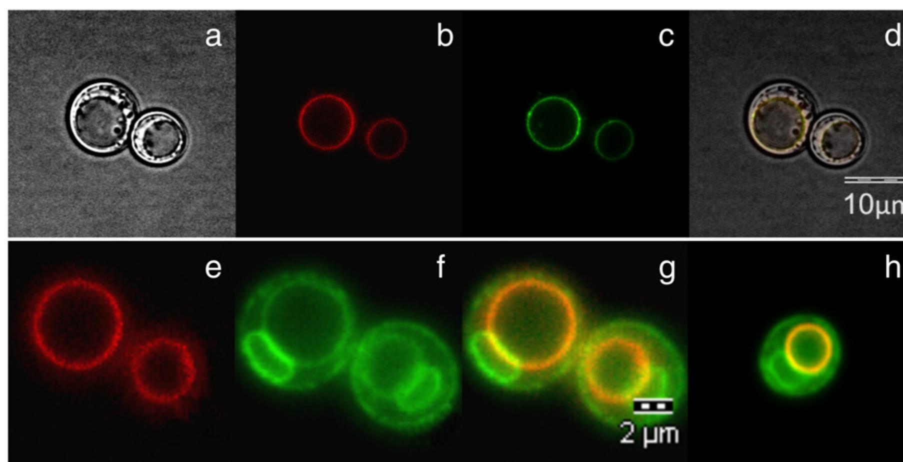

Fig. 3. Subcellular localization of PbtA-eGFP (a–g) and PbtA/A1994G-eGFP (h) fusion proteins in yeasts. The *S. cerevisiae* W303 cells carrying either pGPD-pbtA-egfp or pGPD-pbtA/A1994G-egfp vector were cultured in SD medium to the early growth phase (a–d) or up to the stationary phase (e–h). a) bright-field image; b) and e) visualized vacuoles; c) and f) eGFP fluorescence signal; d) overlay of a), b) and c); g) overlay of e) and f); h) overlay of visualized vacuoles and eGFP fluorescence signal of stationary phase W303 cells bearing pGPD-pbtA/A1994G-egfp vector. Cell^R fluorescence microscope (Olympus) was used to observe fluorescence signal. Vacuoles were stained by FM4-64 dye for 20 min.

E. coli ZntA [36] and *Listeria monocytogenes* CadA [33,37], which are discussed further. Furthermore, the PbtA transporting specificity towards these metal ions was confirmed in our previous study [25].

According to the membrane topology predicted by TMHMM 2.0, PbtA contains seven TMs versus eight or six TMs typical of the most P_{1B}-ATPases. Furthermore, either of the cytoplasmic N-terminal metal binding domains CXXC, CCXSE or $(HX)_n$ widely found in 1B-2 subgroup is also absent in the PbtA sequence [38].

The *pbtA* coding sequence was heterologously expressed in *S. cerevisiae* Cd^{2+}/Pb^{2+}- and Zn^{2+}-hypersensitive strains DTY168 and CM137, respectively, under the control of a constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. The changes in metal resistance of yeast lines expressing *pbtA* were observed when analyzed both on solid and liquid media. Expression of *pbtA* in strain CM137 caused a large increase in zinc resistance. Similarly, DTY168 cells carrying *pbtA* showed restored Pb^{2+}-resistance. In contrast, when *pbtA* was expressed, Cd^{2+}-resistance of Cd^{2+}-hypersensitive strain DTY168 was even more affected. A similar impact of *pbtA* expression was demonstrated in isogenic wild-type *S. cerevisiae* strain W303, where Cd^{2+}-resistance decreased in the same manner as in DTY168. In addition to the results from the metal resistance assays, *pbtA* expression also increased Pb, Zn, and also Cd accumulation in yeasts. When compared with the control, the accumulation rate was the highest for Pb, followed by Cd and Zn. This strong prevalence of Pb accumulation in cells over Cd and Zn is very likely caused by the absence of any known Pb^{2+}-efflux transporters localized in the plasma membrane of *S. cerevisiae* W303 cells which is not the case for Cd^{2+} and Zn^{2+} (see the Introduction section). Pb^{2+} ions present in the cytoplasm of W303 are detoxified only through their accumulation in the vacuole due to action of PbtA and indigenous Pb^{2+}-transporters and are not transported out of the cell efficiently, which would decrease the measured intracellular Pb-accumulation.

As was observed by fluorescence measurements, the PbtA-eGFP protein was successfully incorporated into the cellular membranes in yeast, tending to progressively spread from the tonoplast to plasma membrane during cell aging. As a strong fluorescence signal was observed in the perinuclear region at later stages of growth, we assume that PbtA accumulates in the membranes of ER analogously as described in the work of Wu et al. [39]. The impact of PbtA-eGFP expression on the transition metal resistance in DTY168 and CM137 strains was also studied. Since no phenotypical changes linked to the fusion were observed (data not shown), we assume that the fusion with eGFP did not influence the function and topology of PbtA.

The above-mentioned DKTGT motif is strictly conserved and essential for the function of all P-ATPases since it contains the aspartate residue phosphorylated during the catalytic cycle. Its mutation prevents ATP hydrolysis and, therefore, leads to the loss of transport activity of the P-ATPase [40]. In our study, this aspartate residue at the amino acid position 639 in the PbtA sequence was replaced by glycine. DTY168 and CM137 strains bearing the resultant mutated *pbtA* version exerted the metalloresistance phenotype comparable to that of the negative control strain. A statistically significant increase of metal accumulation was observed only in the case of Pb and with a rate much lower than when the wild-type PbtA was expressed. This evidence gives us a convincing argument that the phenotypical changes in yeasts induced by the expression of the wild-type PbtA protein are caused by its capacity to transport metal ions across membranes where it is embedded rather than a consequence of metal binding capacity of overexpressed imperfectly folded PbtA polypeptide not functioning in the respect of metal transport activity. In addition, localization patterns of the PbtA/A1994G-eGFP fusion protein were identical to that of PbtA-eGFP indicating that the mutated PbtA/A1994G is efficiently expressed in yeast cells and that the A1994G mutation does not affect the PbtA/A1994G subcellular membrane targeting. The phenotype of DTY168 and CM137 cells expressing the PbtA/A1994G-eGFP was also examined and no changes in metalloresistance were observed when compared with both cells bearing pGPD-pbtA/A1994G and p416GPD (data not shown).

Overall, the data obtained indicate that *pbtA* in yeasts encodes for a functioning membrane-localized transporter that takes part in the transport of Pb^{2+}, Zn^{2+} and Cd^{2+} ions.

Considering the subcellular localization observed for PbtA-eGFP fusion protein and the increase in accumulation of all three metals tested in yeast when PbtA was expressed, we assume that PbtA detoxifies Zn^{2+} and Pb^{2+} present in the cytoplasm through their transport into the vacuole and outside the cell. An interesting phenomenon observed was that although yeast cells carrying the *pbtA* gene did confer increased resistance to Zn^{2+} and Pb^{2+}, the Cd^{2+}-sensitive phenotype of DTY168 strain was largely strengthened at the same time. The question of severely increased Cd^{2+}-sensitivity caused by the *pbtA* expression remains to be explained.

To the best of our knowledge, there is not much evidence in the literature regarding the expression of bacterial transition metal transporters in eukaryotes, namely yeasts. According to the study of Gardarin et al. [41], the major target of cadmium toxicity in yeasts is the endoplasmic reticulum (ER). Targeting cadmium to ER renders yeast cells hypersensitive to cadmium. Therefore, it seems reasonable to assume that the observed increased Cd^{2+}-sensitivity of *S. cerevisiae* caused by the expression of *pbtA* could be the consequence of Cd-accumulation and "intoxication" of ER. This conclusion is also in agreement with the observations of localizing the PbtA-eGFP fusion protein in the perinuclear region most likely corresponding to ER. Therefore, PbtA may cause Cd^{2+}-related toxicity through the transport of Cd^{2+} into the lumen of ER. This consideration is entirely in agreement with the findings obtained by Wu [39], where a PbtA homologue P_{1B}-ATPase CadA from *L. monocytogenes*, when expressed in yeast, conferred increased Cd^{2+}-sensitivity and showed a similar localization pattern as PbtA in this study.

In contrast to our findings, however, Lee et al. [42] demonstrate that another PbtA functional homologue P1B-ATPase ZntA from *E. coli* was able to restore the resistance not only to Pb^{2+}, but also to Cd^{2+}, when expressed in *S. cerevisiae*.ZntA was shown to enhance the resistance not only to Pb^{2+}, but also to Cd^{2+}. Unfortunately, the localization of ZntA in yeasts has not yet been studied. However, when expressed in *Arabidopsis thaliana* protoplasts, the ZntA-GFP signal was observed solely in the tonoplast, not in any other membrane systems present within the plant cell. At the same time it is important to bear in mind that all of the three discussed proteins PbtA, CadA and ZntA were expressed without any signal sequence targeting the emerging protein into membranes. In case of PbtA, the predicted protein sequence was screened *in silico* using SignalP [43] for the presence of either prokaryotic or eukaryotic-like targeting sequences, none of which were detected (data not shown). Therefore, it is not likely that the emerging PbtA polypeptide is subjected to any proper signal sequence-driven protein targeting pathway in eukaryotes, which could help explain the observation of a progressive "colonization" of cellular membranes during the cell aging. The absence of any signal sequence-motif is very likely the cause of the discrepancy between our results and those presented by Lee et al. [42] and Wu et al. [39]. In summary, this study indicates that the phenotypical effect of transition metal protein transporters in eukaryotic organisms is very tightly linked with their localization in the subcellular compartment-bordering membrane system.

5. Future perspectives

Recently, extensive effort has been spent on the development of transgenic plants potentially applicable to the remediation of contaminated environment [44–49]. One possibility is represented by the introduction of genes encoding for transition metal protein transporters into convenient plant species leading to their increased toxic metal phytoextraction efficiency and resistance. Although many genetic-determinants mainly from plants and fungi have been intensively studied, only small attention was paid to the genes of bacterial origin [42,50,51]. Nevertheless, the use of bacterial transition metal

transporters seems to be a promising tool for the future enhancement of phytoremediation efficacy, especially if the subcellular localization of employed transporter proteins could be controlled by the utilization of plant indigenous specific membrane-targeting signals, e.g. as described in the works of Sasaki et al. [50] and Kiyono et al. [51]. This study extends the knowledge on the function of such transporters in eukaryotes providing more information helpful for their further application in plant transgenosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.01.023.

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