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Role of VTC4 in stress response and regulation of inorganic polyphosphate levels in yeast

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Abstract: Inorganic polyphosphate (polyP) is an important factor of stress tolerance in microbial cells. In yeast, the major enzyme of polyP biosynthesis is Vtc4, a subunit of the vacuole transporter chaperone (VTC) complex. In this study, we demonstrated that Vtc4 knockout in *Saccharomyces cerevisiae* not only decreased polyP content but also caused shifts in the composition of the intracellular polyP pool and changed the stress tolerance profile. In the mutant *S. cerevisiae*, the level of short-chain acid-soluble polyPs was decreased nearly 10-fold, whereas that of longer acid-insoluble polyPs was decreased only 2-fold, suggesting the existence of other enzymes compensating the production of long-chain polyPs. The *Δvtc4* mutant showed inhibition of Mg^{2+}-dependent phosphate uptake and decreased resistance to alkaline stress but increased tolerance to oxidation and heavy metal ions, especially Mn^{2+}. Quantitative PCR revealed the upregulation of the *DDR2* gene implicated in multiple stress responses and downregulation of *PHO84* encoding a phosphate and Mn^{2+} transporter, which could account for the effects on phosphate uptake and Mn^{2+}-related stress response in the *Δvtc4* mutant. Our study indicates that short-chain polyPs, plays an important role in the regulation of stress response in yeast.

Keywords: inorganic polyphosphate; VTC4; knockout mutant; oxidative stress; gene expression; yeast

1. Introduction

Inorganic polyphosphate (polyP), the linear polymer of orthophosphoric acid, is a universal regulatory biopolymer in living cells [1–4]. PolyP and enzymes of its metabolism are involved in various processes regulating vital activities of the cell. In bacteria, PolyPs are important for stress response and virulence [1,5,6], whereas in the human organism, polyPs play a key role in bone tissue growth and development [7,8], blood coagulation cascade, inflammatory response [9], and signal transduction in astrocytes[10]. Furthermore, PolyP is a component of a specific calcium channel in mitochondrial membranes regulating calcium level and stress response [11].

In yeast, polyPs are involved in the control of the cell cycle [12] and stress response [13–15]. The Vacuole Transporter Chaperone (VTC) complex, which comprises Vtc4 subunit catalyzing polyP biosynthesis, plays an important role in vacuolar membrane fusion and has physical relations with vacuolar H^+-ATPase (V-ATPase), influencing vacuolar H^+ uptake [16,17]. The discovery of the polyP synthase activity of Vtc4 solved the main problem of polyP synthesis in yeast, whose genome does not contain orthologs of bacterial polyphosphate kinases. The mechanism of Vtc4 polyphosphate polymerase activity has been clarified using X-ray crystallography, which has revealed that the Vtc4p$^{189-480}$ fragment contains a long-chain electron-dense domain winding through the tunnel, suggesting that this module generates polyPs from ATP [18]; the catalytic domain faces the cytoplasm and the polymer must pass through the membrane. This Vtc4 fragment demonstrated phosphotransferase activity and could synthesize polyPs in solution from ATP, releasing ADP in the presence of Mn^{2+}. Yeast Δ*vtc4* deletion strains lack the entire vacuolar polyP pool, while *Δvtc1* point mutations targeting the conserved basic residues of transmembrane domains drastically reduce cellular polyP levels [18]. It was proposed that Vtc transmembrane domains of the other proteins in the VTC complex participated in the transport of polyPs across the vacuolar membrane [18]. The VTC exists as two sub-complexes: Vtc4/Vtc3/Vtc1 and Vtc4/Vtc2/Vtc1; the first is located mostly on the vacuole membrane and the second can also be observed in the endoplasmic reticulum and nuclear envelope but is found in the vacuolar membrane under phosphate starvation conditions [19]. The two differently regulated sub-

complexes possibly create polyP pools with different functions. Vtc2, Vtc3, and Vtc4 contain the SPX domain that provides a binding surface for inositol phosphate (InsP) signaling molecules, whose concentrations change depending on the availability of inorganic phosphate (Pi) [20, 21].

Vtc4 is the main enzyme that provides polyP synthesis in fungi. However, yeast cells contain several polyP fractions, which differ in chain length and subcellular localization [22], and it is currently unclear, whether all of them can be synthesized by Vtc4. Although the *vtc4* null mutants of *Saccharomyces cerevisiae* [23] and *Cryptococcus neoformans* [24] have significantly reduced polyP levels, the vtc4 null mutants of *Ustilago maydis* still contain some polyPs [25], suggesting alternative pathways of polyP biosynthesis. In some studies, the methods of polyP extraction and assaying do not allow detection of all cellular polyP fractions [26]. Therefore, more detailed analysis of the polyP profile in *Δvtc4* mutants should be performed for better understanding of the polyP metabolism in eukaryotes. The VTC complex has not been found in mammalian cells [4] and the search of alternative pathways of polyP biosynthesis in yeast may be useful for solving of the problem of polyP biosynthesis in high eukaryotes.

The aim of this study was the analysis of the polyP pool, with the focus on fractions of different chain length, in the *Δvtc4* mutant of the yeast *S. cerevisiae*, a model organism to study polyP metabolism in eukaryotes [1, 27–29]. As polyPs are an important factor of stress response in microorganisms, we also examined the effect of *VTC4* deletion on stress tolerance in yeast. Our results indicate that Vtc4 is predominantly responsible for the level of short-chain acid-soluble polyPs and is involved in the regulation of *S. cerevisiae* responses to oxidative, alkaline, and heavy metal ion stresses.

2. Materials and Methods

2.1. Yeast strains and growth conditions

The *S. cerevisiae* wild-type (WT) strain YSC-1048 and the *Δvtc4* mutant were obtained from the Dharmacon collection. In the experiments with Pi uptake, strains CRN (MATa ade2 his3 ura3 ppn1Δ::CgTRP1) kindly provided by A. Kornberg [30], and PPN1 polyphosphatase-overexpressing CRN/PPN1 (MATa ade2 his3 ura3 ppn1Δ::CgTRP1 transformed with pMB1/PPN1 Sc) [31] were used. Cells were cultured in YPD medium containing 2% glucose, 2% peptone (Sigma-Aldrich), and 1% yeast extract (Pronadisa, Madrid, Spain) at 29 °C and 145 rpm

until the stationary growth stage, harvested by centrifugation at $5,000 \times g$ for 10 min, and washed twice with sterile distilled water.

2.2. PolyP extraction and measurement

PolyPs were extracted as described earlier [22] with minor modifications. To obtain an acid-soluble polyP fraction (polyP1), yeast cell biomass was treated twice with 0.5 M $HClO_4$ at 0 °C for 20 min with stirring. After separation of the supernatant, the remaining biomass was treated twice with saturated $NaClO_4$ solution at 0 °C for 30 min; the supernatant collected after centrifugation represented a salt-soluble polyP fraction (polyP2). Then, a weak alkali-soluble fraction (polyP3) and an alkali-soluble fraction (polyP4) were extracted with 0,1 mM NaOH (pH 10) or 50 mM NaOH, respectively, at 0 °C for 30 min.

PolyPs in the obtained fractions were assayed as acid-labile phosphorus, i.e. Pi released after treatment with 0.5 M HCl at 90 ℃ for 20 min [22]. The last fraction (polyP5) was characterized by the amount of Pi produced after biomass hydrolysis in 0.5 M $HClO_4$ at 90 °C for 20 min.

The Pi content was measured colorimetrically [32] using an EFOS 9505 photometer (Sapphire, Moscow, Russia).

2.3. PolyP electrophoresis

The chain length of polyPs was assessed by electrophoresis as described previously [33] in 24% polyacrylamide gels with 7 M urea; commercial $polyP_{15}$, $polyP_{25}$, and $polyP_{75}$ (Sigma-Aldrich, St. Louis, MO, USA) were used as standards (the numbers indicate the average amount of phosphate residues in the polyP chain). PolyPs were detected by staining the gels with toluidine blue.

2.4. Enzymatic assay of polyPs

For the enzymatic assay, the samples of polyP fractions polyP2 and polyP3 were neutralized to pH 7,0 by HCl aliquotes and incubated with *S. cerevisiae* exopolyphosphatase Ppx1 obtained as described earlier [34]. The reaction mixture containing 0.5 mL of 50 mM Tris-HCl (pH 7.2), 2.5 mM $MgSO_4$, 0.02 mL (~5 U) of Ppx1 preparation, and 0.1 mL of polyP extracts was incubated at 30 °C for 2 h with shaking, and the released Pi was assayed as previously described [32]. Commercial $polyP_{188}$ (Monsanto, Creve Coeur, MO, USA) was used as control.

2.5. Determination of yeast sensitivity to peroxide, alkali, and heavy metal ions

Yeast samples normalized by cell concentration ($0.5 \cdot 10^7$ cell/ mL) were added to the wells of sterile plates containing the YPD medium and different concentrations of $Cd(CH_3COO)_2 \cdot 2H_2O$, $MnSO_4$, $CoSO_4$, $NiSO_4$, hydrogen peroxide, or KOH, cultured for 24 h, and the optical density was measured at 594 nm using an EFOS photometer.

2.6. Pi uptake

Freshly harvested yeast cells (~ 55 mg wet biomass) were incubated in 0.75 mL of MiliQ water containing 110 mM glucose and 1 mM K_2HPO_4 and supplemented or not with 5 mM $MgSO_4$, at 30 °C with shaking (850 rpm) in ThermoMixer (Eppendorf, Hamburg, Germany). After 60 min, the cells were centrifuged at $5,000 \times g$ and Pi was assayed in supernatants by the colorimetric method with malachite green [34].

2.7. Quantitative PCR

Yeast were grown in 25 mL of YPD medium in 250-mL flasks at 28 °C, and biomass from 10 mL of culture was harvested after 36-h growth (stationary phase). Total RNA was extracted using the acid hot phenol method [35]; two biological replicates were used. RNA quality was assessed by electrophoresis in 1.5% agarose TBE gels. After treatment of RNA with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) followed by purification using the RNA CleanUp Kit (Evrogen, Moscow, Russia), cDNA was synthesized using the cDNA RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) and random hexamer primers.

Quantitative real-time (qRT)-PCR was carried out in a CFX96 Cycler-RealTime PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR Green 2,5 Master Mix (Syntol, Moscow, Russia) and *S. cerevisiae* gene-specific primers (Supplemental Table S1) designed with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primerblast/) and synthesized by Evrogen (Moscow, Russia). The reactions were performed with 2.5 ng of cDNA at the following cycling conditions: initial denaturation at 95 °C for 5 min and 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 40 s. To normalize gene expression levels, the *S. cerevisiae ALG9* gene was used as reference [36]. The qRT-PCR results were statistically analyzed with Graph Pad Prism version 7.02 (GraphPad Software Inc., San Diego, CA, USA;

https://www.graphpad.com/scientific-software/prism/) and gene expression levels were calculated relative to *ALG9* expression using the $2^{-\Delta\Delta CT}$ method [37].

2.8. Statistics

The experiments were performed in triplicate and the results are presented as the mean with standard deviation (Excel). The experiment with electrophoresis was repeated twice.

3. Results

3.1. VTC4 knockout mutant contains polyPs

The question of whether *S. cerevisiae* cells lacking polyP synthase Vtc4 can still produce polyPs remains unresolved because of differences in polyP extraction methods [23–26]. We used a polyP extraction method that allowed obtaining separate fractions of polyPs with different chain length [22]. Comparison of the polyP content in the *Δvtc4* and WT strains showed that the short-chain polyP1 fraction was decreased 10-fold, whereas longer chain polyP2 and polyP3 fractions decreased only 2-fold in *Δvtc4* compared to parental cells, and polyP4 and polyP5 fractions containing the longest polyPs of yeast cells [22, 38] did not differ between the strains (Figure 1A). Furthermore, the Pi level in the *Δvtc4* strain was lower than that in the WT strain (Figure 1A). These results indicated that *Δvtc4* cells still had a considerable amount of polyP2 and polyP3 with longer chains.

The presence of polyPs in the fractions was confirmed by electrophoresis and enzymatic assays. Electrophoregrams revealed characteristic bands stained by toluidine blue in polyP1, polyP2, and polyP3 fractions (Figure 1B). The polyP chain length in these fractions was similar for WT and*Δvtc4* cells (Figure 1B) and corresponded to that observed earlier for *S. cerevisiae* [22, 38]. PolyPs in polyP2 and polyP3 fractions were hydrolyzed by Ppx1 polyphosphatase; however, in both strains the hydrolysis was incomplete (Figure 1C). Incomplete hydrolysis (88%) was also observed for commercial polyP with the average chain length of 188 phosphate residues. The inaccessibility of polyPs from biological sources for enzymatic hydrolysis has been reported earlier [30], but the reason is still unclear. One explanation can be the presence of Ca^{2+} or Fe^{2+} in polyP preparations obtained from the yeast cells, which could inhibit Ppx1 activity [39].

Overall, these results indicated that the *Δvtc4* knock-out mutant could still synthesize a considerable amount of polyPs.
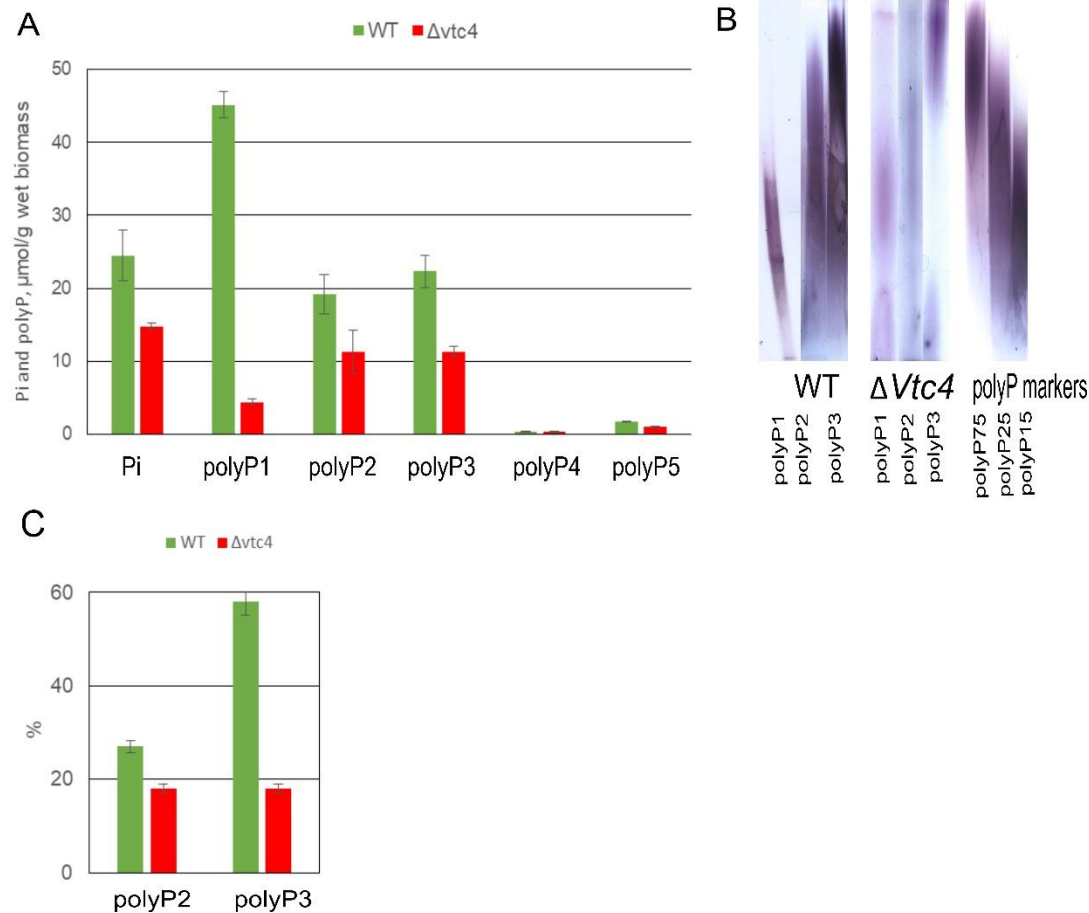
Figure 1. Pi and polyP in WT and *Δvtc4* S. cerevisiae strains. (A) Amount of Pi and polyPs in different fractions measured by acid hydrolysis. (B) A representative electrophoregram of polyP1, polyP2, and polyP3 fractions of WT and *Δvtc4 S. cerevisiae* strains. PolyP markers were commercial polyP (Sigma, USA) with average chain length of 75 (polyP75), 25 (polyP25) and 15 (polyP15) phosphate residues. The experiment was repeated twice with similar results. (C) Pi released from polyPs of polyP2 and polyP3 fractions after cleavage by Ppx1 polyphosphatase, % of Pi assayed as labile phosphorus in the fractions, see Fig. 1A.

3.2. Pi uptake by WT and *Δvtc4* strains

Considering the reduction of Pi and polyP levels in the mutant strain, we compared Pi uptake in the *Δvtc4* strain with that in the WT strain in the presence of Mg^{2+} ions, which should stimulate the process through the effect on high-affinity phosphate transporter Pho84 involved in both Pi and manganese uptake in *S. cerevisiae* [40, 41]. The results indicated that there was no stimulation of Pi uptake by Mg^{2+} in the *Δvtc4* strain (Figure 2A). The same experiment performed in the CRN

strain and the CRN/PPN1 strain overexpressing polyphosphatase Ppn1 [31] revealed that Ppn1 overproduction reduced Pi uptake (Figure 2B).
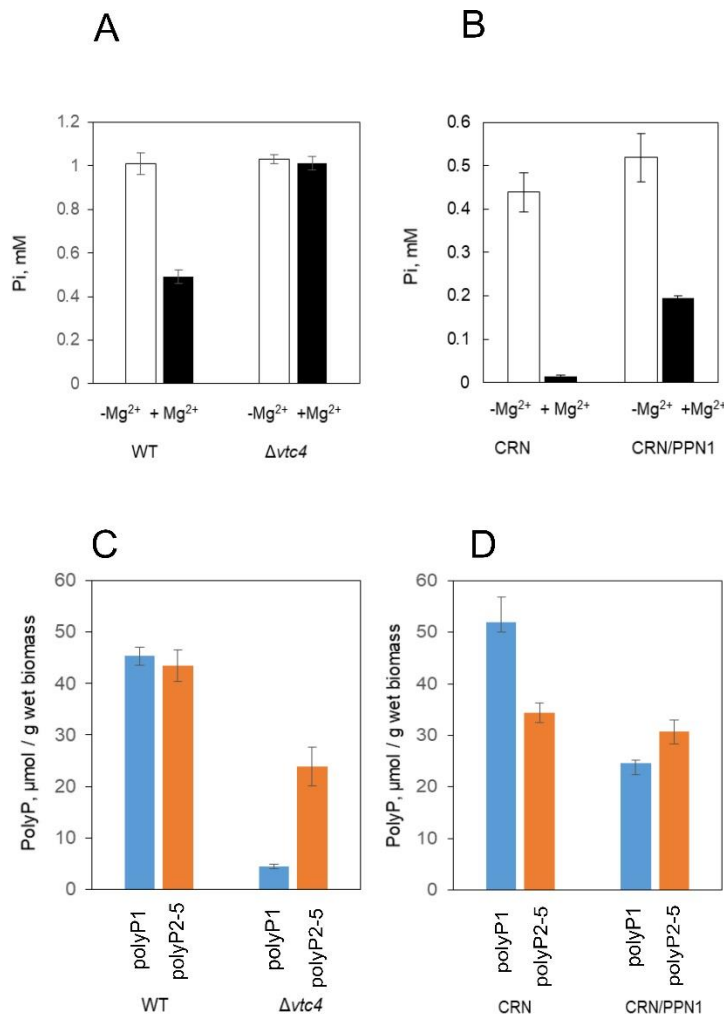

Figure 2. Pi uptake and polyP content in the cells of WT, *Δvtc4*, CRN, and CRN/PPN1 strains of *S. cerevisiae*. (A, B) Pi uptake by WT and *Δvtc4* strains (A) and CRN and CRN/PPN1 strains (B) measured by assessing Pi remaining in the incubation medium. The stationary grown cells of WT, *Δvtc4*, CRN, and CRN/PPN1 strains were incubated in water containing 110 mM glucose and 1 mM K_2HPO_4 and supplemented or not with 5 mM MgSO4. (C, D) Content of short-chain (polyP1) and long-chain (a sum of polyP2, polyP3, polyP4 and polyP5 indicated as polyP2-polyP5) polyPs in WT and Δvtc4 strains (C) and CRN and CRN/PPN1 strains (D).

So, the *Δvtc4* strain and CRN/PPN1 strain in which short-chained polyP1 were significantly decreased compared to the WT strain (Figure 2C and D) demonstrated reduced Pi uptake in the presence of Mg^{2+}. Earlier we observed the decrease of *PHO84* gene expression in the CRN/PPN1 strain [14]. These results suggest that the decrease in short-chain polyPs may serve as a signal to

decrease Pi transport, possibly through downregulation of *PHO84* gene expression or Pho84 transporter activity.

3.3. Stress tolerance

PolyP is an important factor in stress resistance of microorganisms. Vtc4 is involved in the function of vacuoles [16, 17], which play a significant role in stress resistance of yeast [42]. Therefore, we compared the sensitivity of WT and *Δvtc4* strains to stressful conditions such as high pH, hydrogen peroxide, and heavy metals. The *Δvtc4* strain was more sensitive to alkaline stress, as evidenced by total growth cessation in the presence of 60 mM KOH, whereas the growth of the WT strain at this concentration was inhibited only by 50% (Figure 3A). However, unexpectedly the mutant strain appeared to be more resistant to the other tested stressful conditions (Figure 3B-F). The most pronounced difference between the strains was observed in the presence of Mn^{2+}. Thus, the growth of the WT strain was already markedly inhibited at 2 mM $MnSO_4$, whereas in the *Δvtc4* strain the same level of growth inhibition was observed at 7 mM $MnSO_4$ (Figure 3E).
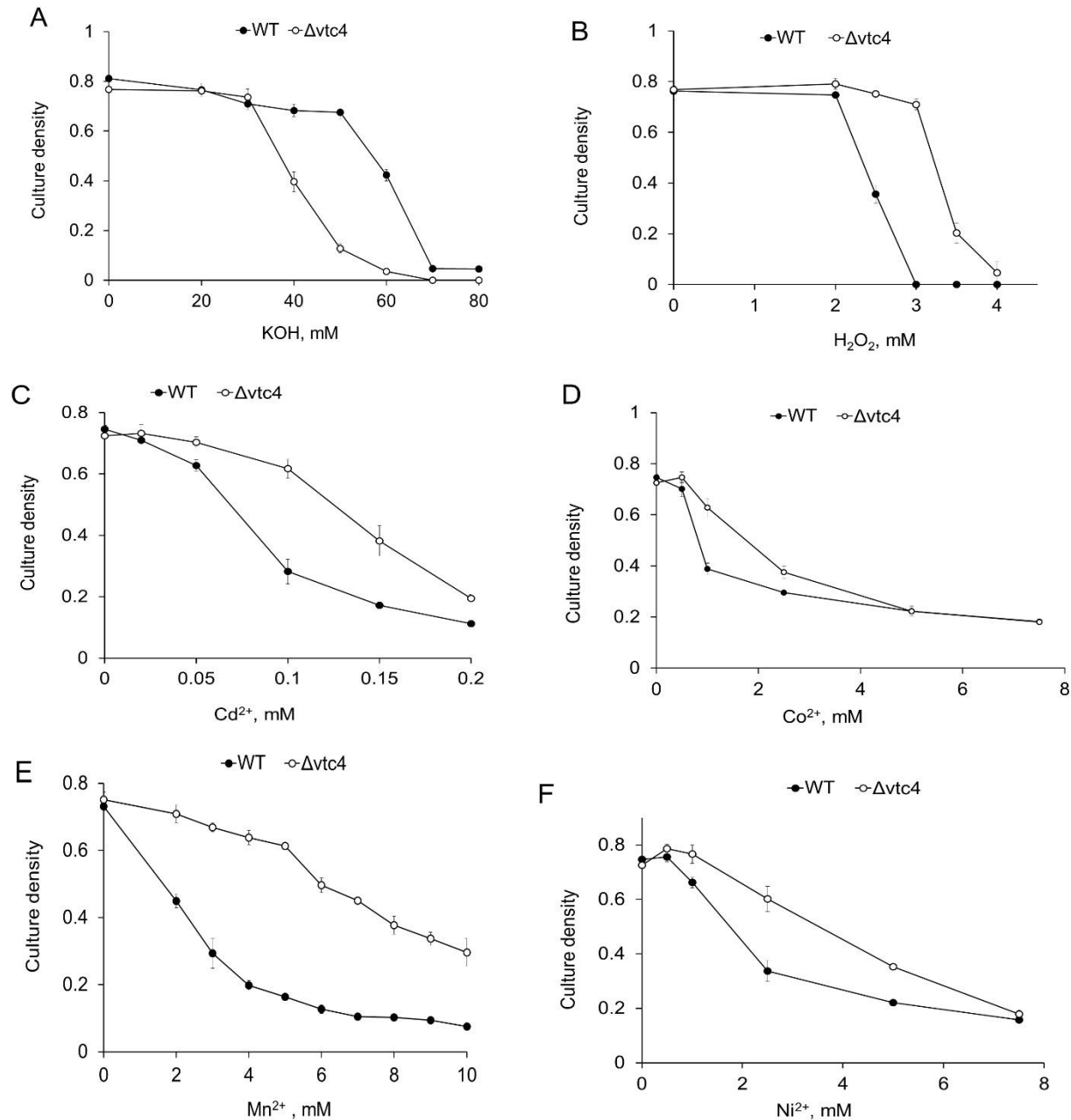
Figure 3. Effects of alkali, hydrogen peroxide, and heavy metals on the growth of WT and *Δvtc4 S. cerevisiae* strains.

3.4. Real-time PCR

The resistance to manganese and peroxide stresses observed in the *Δvtc4* strain was also characteristic for the Ppn1-overexpressing CRN/PPN1 strain [14], in which it was accompanied by changes in the transcription of genes associated with response to external stimulus, plasma

membrane organization, and oxidation/reduction; furthermore, manganese tolerance in the CRN/PPN1 strain was correlated with *PHO84* downregulation. Therefore, we analyzed the expression of genes differentially regulated in the CRN/PPN1 strain, in the WT and *Δvtc4* strains (Figure 4). The results indicated that, similar to the CRN/PPN1 strain, the *DDR2* gene encoding the multi-stress response factor activated by a variety of xenobiotic agents and environmental or physiological stresses (https://www.yeastgenome.org/locus/S000005413) was upregulated in the *Δvtc4* strain. However, the expression of the *PHM7* gene, which encodes a putative transport protein (https://www.yeastgenome.org/locus/S000005444) and which was upregulated in the CRN/PPN1 strain, did not differ between the *Δvtc4* and WT strains. The *PHO5* and *PHO84* genes encoding an acid phosphatase and Pi transporter Pho84, respectively, were downregulated, although in case of Pho84 the effect was less pronounced; the expression of these two genes was also decreased in the CRN/PPN1 strain. The observed transcriptional effects of *VTC4* deletion were consistent with the increased tolerance to oxidation and manganese and with the decreased Mg^{2+}-dependent Pi uptake in the *Δvtc4* strain.
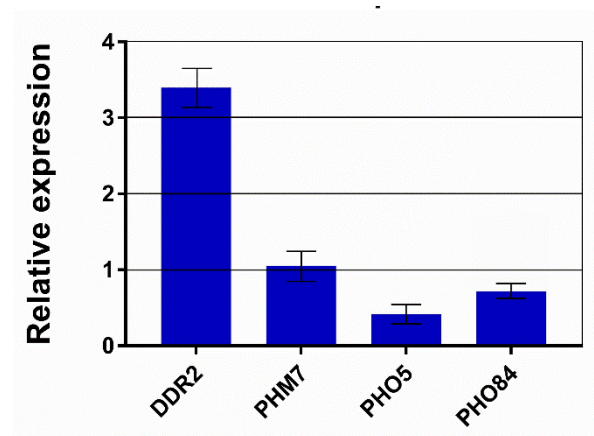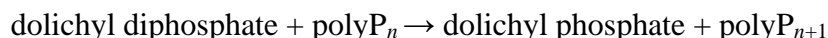

Figure 4. Differential expression of the selected target genes between the *Δvtc4* and WT strains. Data are means ± SD, n = 3.
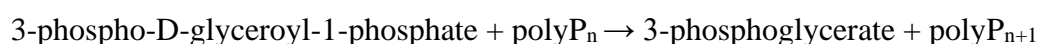
4. Discussion

Analysis of the polyP content and stress responses in the *Δvtc4* mutant revealed two physiological effects, which are important for further understanding of the polyP metabolism in eukaryotes. First, the Vtc4 knockout mutant still contained a significant amount of polyPs,

suggesting that other enzymes could also perform polyP synthesis in fungal cells. One such enzyme is dolichyl diphosphate:polyP phosphotransferase (EC 2.7.4.20) catalyzing the reaction:

dolichyl diphosphate + $polyP_n \rightarrow$ dolichyl phosphate + $polyP_{n+1}$

This activity was found in the membrane fraction of yeast cells extracted with Triton X-100 [43]. The specific activity of the solubilized preparation was 20-fold higher than that in the protoplast lysate. The catalytic activity of dolichyl diphosphate:polyP phosphotransferase was metal-dependent, showing the maximum in the presence of Mg^{2+} or Ca^{2+}, and was inhibited by EDTA.

The other enzyme is 3-phospho-D-glyceroyl-phosphate:polyphosphate phosphotransferase (1,3-diphosphoglycerate-polyphosphate phosphotransferase; EC 2.7.4.17) catalyzing the reaction:

3-phospho-D-glyceroyl-1-phosphate + $polyP_n \rightarrow$ 3-phosphoglycerate + $polyP_{n+1}$

This activity was first observed in the adenine-deficient *Neurospora crassa* mutant, where concentrations of ATP and other adenyl nucleotides were sharply reduced [44], and was also detected in the WT *N. crassa* strain and some other bacterial species, but at a much lower level. The *Δvtc4* mutant is a convenient model for the search of alternative polyP biosynthesis enzymes and their cellular localization in future studies.

The second feature is the increased resistance of the *Δvtc4* mutant to stresses caused by oxidation and heavy metal ions. In this respect, the *Δvtc4* mutant is similar to the Ppn1-overexpressing strain, which also exhibits a reduced content of short-chain polyPs (acid-soluble polyP1 fraction). The observed increase in the expression of the *DDR2* gene encoding one of the important stress response regulators and a slight decrease in that of *PHO8*, the transporter of Pi and bivalent metal ions, indicate a possible similarity of stress response mechanisms in the Vtc4-deficient and Ppn1-overexpressing strains. It is suggested that short-chain polyPs serve as signaling molecules and their decrease leads to the activation of stress response genes, which can be associated with the antioxidant properties of polyPs [45]. With regard to the reduced resistance of the *Δvtc4* mutant to the alkaline stress, it is consistent with the notion of the important role of short-chain polyPs in cellular pH homeostasis. Thus, it was shown that a strain overexpressing endopolyphosphatase Ppn2, which cleaves long-chain polyPs into shorter ones, had a significantly higher content of short-chain polyPs and was more resistant to the alkaline stress than the parental strain [46].

In conclusion, our results indicate that Vtc4 polyphosphate synthase is responsible for the level of short-chained polyP and a part of long-chained polyP, suggesting the presence of

alternative biosynthetic pathways for long-chain polyPs in yeast. The increased tolerance of the Δvtc4 mutant to peroxide and heavy metals could be attributed to the activation of stress-response genes due to the decrease in short-chain polyPs. This study provides further evidence of the distinct biological functions of short-chain and long-chain polyPs, indicating a multifunctional role of polyPs in yeast.

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