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V-ATPase dysfunction suppresses polyphosphate synthesis in *Saccharomyces cerevisiae*

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Abstract The yeast *Saccharomyces cerevisiae* accumulates the high levels of inorganic polyphosphates (polyPs) performing in the cells numerous functions, including phosphate and energy storage. The effects of vacuolar membrane ATPase (V-ATPase) dysfunction were studied on polyP accumulation under short-term cultivation in the P_i–excess media after P_i starvation. The addition of bafilomycin A1, a specific inhibitor of V-ATPase, to the medium with glucose resulted in strong inhibition of the synthesis of long-chain polyP and in substantial suppression of short-chain polyP. The addition of bafilomycin to the medium with ethanol resulted in decreased accumulation of high-molecular polyP, while the accumulation of low-molecular polyP was not affected. The levels of polyP synthesis in the mutant strain with a deletion in the *vma2* gene encoding a V-ATPase subunit were significantly lower than in the parent strain in the media with glucose and with ethanol. The synthesis of the longest chain polyP was not observed in the mutant cells. The synthesis of only the low-polymer acid-soluble polyP fraction occurred in the cells of the mutant strain. However, the level of polyP1 was nearly tenfold lower than compared to the cells of the parent strain. Both bafilomycin A1 and the mutation in vacuolar ATPase subunit *vma2* lead to a considerable decrease of cellular polyP accumulation. Thus, the defects in $\Delta\mu H^+$ formation on the vacuolar membrane resulted in the decrease of polyP biosynthesis in *S. cerevisiae*.

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Introduction

High-polymeric inorganic polyphosphates (polyPs) are linear polymers containing a few to several hundred orthophosphate residues and performing numerous functions in the cells, including phosphate and energy storage, sequestration of cations, formation of membrane channels, involvement in cell envelope formation and function, gene activity control, regulation of enzyme activities, stress response, and stationary phase adaptation (Kulaev et al. 2004; Rao et al. 2009; Achbergerová and Nahálka 2011; Orell et al. 2012). The yeast *Saccharomyces cerevisiae* is a good model for investigation of the metabolism of these biologically active polyanions. However, polyP biosynthesis in these microorganisms has been little studied. *S. cerevisiae* lacks polyphosphate kinase, the enzyme responsible for the synthesis of the most part of polyP in bacteria (Rao et al. 2009). Only two enzymes of polyP synthesis in *S. cerevisiae* are known at present: dolychylpyrophosphate: polyphosphate transferase responsible for the synthesis of a minor part of yeast cell polyP localized mainly in the cell wall (Kulaev et al. 1987) and Vtc4 protein, a transport chaperone localized in the vacuolar membrane (Hothorn et al. 2009).

The important role of vacuoles in polyP metabolism was postulated rather long ago (Durr et al. 1979; Ogawa et al. 2000). However, it is still little understood. The vacuolar membrane ATPase (V-ATPase) is important for many cellular processes in yeasts (Bouillet et al. 2012; Zhang and Rao 2012; Marshall et al. 2012). The mutation in the *vma2* gene encoding the protein B of subunit V1 of V-ATPase (Milgrom et al. 2007) decreases the content of polyP in *S. cerevisiae* during cultivation on glucose (Tomaschevsky et al. 2010). This result suggests an important role of vacuolar membrane energization in polyP biosynthesis in *S. cerevisiae*. However, the processes of polyP accumulation in yeast cells under cultivation on different carbon sources are

characterized by distinctive features. Glucose and ethanol are indicative carbon sources which differ in main mechanism of energy supply in *S. cerevisiae* cells: under glucose consumption, it is glycolysis, while under ethanol consumption, it is oxidative phosphorylation. Previously, we have revealed that the accumulation of long-chain and short-chain polyPs increases and decreases, respectively, under cultivation on ethanol (Vagabov et al. 2008). The question arises whether this effect is associated with the changes in polyP biosynthetic pathways under cultivation on ethanol compared to cultivation on glucose.

The goal of this work was to find out the effects of V-ATPase dysfunction on polyP biosynthesis in *S. cerevisiae* under cultivation in media with glucose and ethanol.

Materials and methods

Research objects and culture conditions The objects of research were the wild strain of *S. cerevisiae* VKM Y-1173 (All-Russian Collection of Microorganisms, Russian Academy of Sciences), the mutant strain BY 4741 vma2Δ (a deletion in the *vma2* gene), and its parent strain BY 4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). The strains BY 4741 and BY 4741 vma2Δ were kindly provided by Dr. P. Kane, SUNY Upstate Medical University, USA (Milgrom et al. 2007). The BY 4741 and BY 4741 vma2Δ yeast cultures were maintained on YPD agar slants; the strain VKM Y-1173 was maintained on Wort agar slants.

For the experiments, the yeast was cultivated in liquid phosphate-containing (+P) and phosphate-free (−P) media. The growth of the strains BY 4741 and BY 4741 vma2Δ is shown in Fig. 1. The growth of the strain VKM Y-1173 was similar to BY 4741 (data not shown). The cultures were grown in shakers at 29 °C in the flasks containing 200 ml of the liquid medium supplemented with 2 % glucose as a carbon source at 120 rpm or 50 ml of the medium with 1 % ethanol at 200 rpm.

The (+P) medium contained (in gram per liter): $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 1.025; NaCl, 0.1; $CaCl_2$, 0.1; KH_2PO_4, 0.85; K_2HPO_4, 0.15; $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 0.25×10^{-3}; yeast extract, 2; and trace elements (Vagabov et al. 2008) for cultivation of the strain VKM Y-1173. For cultivation of the strains BY 4741 and BY 4741 vma2Δ, the same medium was supplemented with 60 mg/L of histidine, methionine, and uracyl and with 90 mg/L of leucine. For the cultivation of BY 4741 vma2Δ strain, the pH values of all media used were adjusted to 5.0 by adding 50 mM succinate–NaOH buffer.

The (−P) medium contained (in gram per liter): $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 1.025; NaCl, 0.1; $CaCl_2$, 0.1; KCl, 0.6; $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 0.25×10^{-3}; phosphate-free yeast extract, 2; and trace elements. The phosphate-free yeast extract was obtained as described (Rubin 1973).

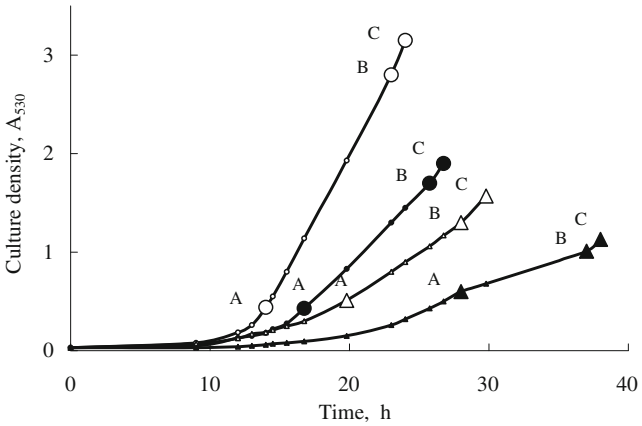

Fig. 1 The growth of the strains BY 4741 and BY 4741 vma2Δ of *S. cerevisiae*. The strains were grown in (+P) media to a culture density of 0.4–0.6 (*point A*). Then, the cells were precipitated at 3,000×*g* and washed with the (−P) medium. The cells were re-inoculated in the (−P) medium and cultivated for 9 h (strains BY 4741 and BY 4741 vma2Δ) or for 7 h (strain VKM Y-1173) (*point B*). After that, the cells were precipitated at 3000×*g*, re-inoculated in the (+P) medium, and cultivated for 1 h (strains BY 4741 and BY 4741 vma2Δ) or for 0.5 h (strain VKM Y-1173) (*point C*). *White circle* strain BY 4741, the medium with glucose; *black circle* BY 4741 vma2Δ, the medium with glucose; *triangle* strain BY 4741, the medium with ethanol; *black up-pointing triangle* BY 4741 vma2Δ, the medium with ethanol

The yeast strains were grown in (+P) media to a culture concentration corresponding to 0.4–0.6 (A_{530}, measured in a 3.07-mm cuvette, point A in Fig. 1). Then, the cells were precipitated at 3,000×*g* and washed with the (−P) medium. The cells were re-inoculated in the (−P) medium and cultivated for 9 h (strains BY 4741 and BY 4741 vma2Δ) or for 7 h (strain VKM Y-1173) (point B, Fig 1). The times of cultivation in the (−P) medium were selected in preliminary experiments to obtain minimal polyP levels in the cells.

After that, the cells were precipitated at 3,000×*g*, re-inoculated in the (+P) medium, and cultivated for 1 h (strains BY 4741 and BY 4741 vma2Δ) or for 0.5 h (strain VKM Y-1173) (point C, Fig 1).

The strain VKM Y-1173 was cultivated for 0.5 h in the absence (control) or presence of 0.375 μmol/L bafilomycin A1. Biomass was harvested by centrifugation at 3,000×*g*, washed twice with cold distilled water, and used for the assay.

Table 1 Total polyP content (in micromole P per gram dry biomass) in the cells of *S. cerevisiae* grown under P_i starvation (Fig. 1, point B) in the medium with glucose or ethanol

Strain	Glucose	Ethanol
VKM Y-1173	32±3.3	48±6.1
BY 4741	74±5.4	69±4.8
BY 4741 vma 2Δ	57±2.9	62±3.4

Table 2 The effect of bafilomycin A1 on polyP content (in micromole P per gram dry biomass) in separate fractions. The P_i-starved cells of S. *cerevisiae* VKM Y-1173 were cultivated for 0.5 h in (+P) media with glucose or ethanol (Fig. 1, point C)

PolyP fraction	polyP1	polyP2	polyP3	polyP4	polyP5
Glucose					
Control	492±42.1	199±18.3	252±17.7	40±3.6	87±8.3
+ Bafilomycin A1 (0.375 μmol/L)	218±20.2	75±5.5	20±2.4	6±1.0	7±0.9
Ethanol					
Control	135±10.8	100±9.1	315±27.3	59±4.4	389±31.8
+ Bafilomycin A1 (0.375 μmol/L)	198±15.2	105±8.5	220±18.7	30±2.1	215±16.9

Polyphosphate extraction and assay PolyP fractions were obtained and the polyP content was quantified as described earlier (Vagabov et al. 2008). The following polyP fractions were obtained: acid-soluble fractions (polyP1), salt-soluble fractions (polyP2), two alkaline-soluble fractions (polyP3 and polyP4), and a hot chlorine extract fraction (polyP5). P_i was assayed by the method of Heinonen and Lahti (1981).

Polyphosphate chain lengths were determined by electrophoresis in 20 % polyacrylamide gel prepared in 200 mmol/L Tris–borate buffer, pH 8.3, with 7 mol/L urea (Kumble and Kornberg 1996). Commercial polyphosphates with different average chain lengths (Sigma and Monsanto, USA) were used as markers.

ΔpH formation on membrane of isolated vacuoles Vacuoles were obtained as described earlier (Lichko and Okorokov 1984). Formation of the ΔpH on membrane of isolated vacuoles was registered by 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching in a Hitachi MPF-4 microfluorimeter (Japan) as described earlier (Lichko and Okorokov, 1984). The incubation medium contained (in millimole per liter): glucitol, 100; MES, 10; ACMA, 0.125; and $MgSO_4$, 50; and pH was adjusted to 7.2 with NaOH. The reaction was started by adding 2 mmol/L ATP and $MgSO_4$.

ATP assay ATP was extracted from the cells by adding dimethyl sulfoxide (0.2 ml per 25–50 mg of wet biomass). ATP was assayed with luciferin–luciferase assay kit (Sigma, USA) using a luminometer 1250 (LKB, Sweden).

All experiments were performed three times and the average values are presented. The χ^2 values were no more than 10 % in all experiments presented.

Results and discussion

Bafilomycin A1, the specific inhibitor of V-ATPase, suppresses $\Delta\mu H^+$ formation on the vacuolar membrane (Bowman et al. 1988). We have studied the effect of bafilomycin on polyP synthesis in the cells of *S. cerevisiae* VKM Y-1173. Phosphate-starved cells (Fig. 1, point B) contained low levels of polyP (Table 1). These cells were re-inoculated in a fresh medium with 9 mmol/L P_i supplemented with glucose or ethanol. The cells accumulated polyP after 0.5-h cultivation (Fig. 1, point C). Table 2 shows the levels of polyP in the fractions different in chain length. The average chain lengths of polyP were 15, 25, 65, 75, and more than 200 phosphate residues for polyP1, polyP2, polyP3, polyP4, and polyP5, respectively. These average chain lengths did not depend on the carbon source, which is in agreement with the data obtained previously for this strain (Vagabov et al. 2008), and did not vary in the presence of bafilomycin. More short-chain polyP (polyP1 and polyP2) and less long-chain polyP (polyP3, polyP4, and polyP5) were synthesized in the medium with glucose, while more long-chain polyP and less short-chain polyP were synthesized in the medium with ethanol (Table 2). The addition of bafilomycin to the medium with glucose resulted in strong inhibition of the synthesis of long-chain polyP3, polyP4, and polyP5 and substantial suppression of short-chain polyP1 and polyP2 (Table 2). The addition of bafilomycin to the medium with ethanol resulted in the decrease of the quantity of high-molecular fractions polyP3, polyP4, and polyP5, while the accumulation of low-molecular fractions polyP1 and polyP2 was not inhibited (Table 2).

Table 3 The effect of bafilomycin A1 on total polyP content (in micromole P per gram dry biomass) and ATP content (in micromole ATP per gram dry biomass) in the cells of S. *cerevisiae* VKM Y-1173. The P_i-starved cells were cultivated for 0.5 h in (+P) media with glucose or ethanol (Fig. 1, point C)

Cultivation	P_i		ATP		Sum polyP	
	Glucose	Ethanol	Glucose	Ethanol	Glucose	Ethanol
Control	94±8.9	84±8.2	7±0.8	12±1.0	1070±75.3	998±71.9
+ Bafilomycin A1 (0.375 μmol/L)	134±11.8	82±7.5	31±2.4	14±1.5	326±22.7	768±58.1

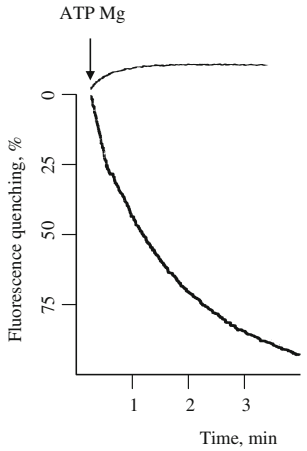

Fig. 2 Formation of the ΔpH on membrane of isolated vacuoles ($\Delta F/F$—the relative fluorescence quenching). The incubation medium contained (in millimole per liter): glucitol, 100; MES, 10; ACMA, 0.125; and $MgSO_4$, 50; pH was adjusted to 7.2 with NaOH. The reaction was started by adding 2 mmol/L ATP and $MgSO_4$. *Lower curve*–vacuoles from the cells of parent strain BY 4741, *upper curve*–vacuoles from the cells of strain BY 4741 vma2Δ

The ATP level increased in the cells grown on glucose in the presence of bafilomycin (Table 3). It may be caused by the decrease of ATP consumption by V-ATPase. The enhancement of P_i level correlated with the decrease in polyP biosynthesis (Table 3). In the cells grown on ethanol, bafilomycin had no effect on P_i and ATP levels and the suppression of polyP synthesis was less pronounced. The less pronounced effect of bafilomycin on the cells cultivated in the medium with ethanol may be explained by the lower penetration of the inhibitor across the cytoplasmic membrane. The cultivation on ethanol changes the lipid composition and decreases the cytoplasmic membrane permeability (Herve et al. 1994).

Another approach to revealing the role of the $\Delta\mu H^+$ on the vacuolar membrane in polyP biosynthesis is to use mutants in the genes encoding V-ATPase subunits. Taking into account possible differences in polyP levels in different yeast strains, we used the parent strain BY-4741 as a control. We have observed the ATP-dependent formation of ΔpH on the vacuolar membrane of the parent strain. The ΔpH was not formed on the vacuolar membrane of the mutant strain vma2Δ (Fig. 2).

Phosphate-starved cells of the parent and mutant strains (Fig. 1, point B) contained similarly low levels of polyP (Table 1). We have compared the levels of polyP synthesis in the parent strain BY-4741 and the mutant strain BY-4741 vma2Δ during 1-h cultivation (Fig. 1 point C) in the media with glucose or ethanol. The cells of parent strain accumulated polyP in both media (Table 4). The level of polyP synthesis in the mutant strain was significantly lower than in the parent strain in the media with glucose and with ethanol (Table 4). The synthesis of the most long-chain polyP (fractions polyP4 and polsyP5) was not observed, and the synthesis of polyP3 and polyP2 was insignificant. The synthesis of only the low-polymer fraction polyP1 was observed in the cells of the mutant strain (Table 4). However, the level of polyP1 was yet nearly tenfold lower than that in the cells of the parent strain.

The Vtc4 protein, a transport chaperone localized in the vacuolar membrane, is known to be a polyphosphate synthetase (Hothorn et al. 2009). Up to now, no experimental model yielding the high level of polyP biosynthesis in cell-free extracts or preparations of vacuoles has been described. We have failed to obtain polyP synthesis in the preparation of isolated vacuoles (data not shown). So, we have used the native cells of *S. cerevisiae*, for which a model for the study of polyP synthesis has been developed (Vagabov et al. 2008).

Two approaches are commonly used in such studies: the specific inhibitors in a valid concentration and mutations in genes important for the process. The effect bafilomycin A1 on polyP synthesis in the cells of *S. cerevisiae* under glucose consumption was analyzed earlier (Trilisenko et al. 2003). Bafilomycin at concentration of 0.05 μmol/L according to reference (Bowman et al. 1988) exhibited a little effect on polyP biosynthesis. Only the level of polyP3 decreased for ~30 % (Trilisenko et al. 2003). In our preliminary experiments, an effective concentration of bafilomycin A1

Table 4 The polyP content (in micromole P per gram dry biomass) in separate fractions obtained from the cells of S. *cerevisiae*. The P_i-starved cells were cultivated for 1 h in (+P) media with glucose or ethanol (Fig. 1, point C)

Strain	Cultivation medium	polyP1	polyP2	polyP3	polyP4	polyP5
Glucose						
BY 4741, parent strain	−P	19±3.1	12±1.8	27±0.8	6±0.1	10±0.4
	+P	183±7.8	62±3.3	138±6.1	10±2.2	18±1.5
BY 4741 vma 2Δ	−P	6±1.0	12±1.5	27±0.6	5±0.5	8±1.0
	+P	23±1.1	14±1.3	30±1.3	6±0.4	8±0.8
Ethanol						
BY 4741, parent strain	−P	8±0.9	17±1.6	25±1.1	9±1.3	15±1.4
	+P	95±4.7	169±12.2	136±10.5	10±1.1	28±1.9
BY 4741 vma 2Δ	−P	4±0.6	16±1.3	19±1.7	15±1.5	7±0.4
	+P	13±1.0	19±1.4	22±1.8	15±1.3	7±0.5

(0.375 μmol/L) was selected. It had no effect on cell growth and exopolyphosphatase activity (not shown). A pronounced inhibitory effect of bafilomycin A1 on polyP accumulation was revealed at first in this study (Table 2). The effects of bafilomycin were firstly compared under consumption of two different carbon sources.

The decrease of polyP content in mutant in the vacuolar ATPase subunit vma2 was observed earlier under cultivation on glucose (Tomaschevsky et al. 2010). This effect was confirmed under cultivation on ethanol in this work. Besides, a more adequate model for polyP biosynthesis assay was used. It should be noted that exopolyphosphatase activities in cell homogenates of the parent strain and vma2Δ strain were similar (~10 mU/mg protein).

The disturbance of $\Delta\mu H^+$ on the vacuolar membrane resulted in the decrease of polyP biosynthesis in *S. cerevisiae* independent of the prevalence of glycolysis (cultivation in the medium with glucose) or oxidative phosphorylation (cultivation in the medium with ethanol). Probably, polyP biosynthesis by polyphosphate synthetase Vtc4 localized in the vacuolar membrane (Hothorn et al. 2009) needs $\Delta\mu H^+$ at this membrane.

The data obtained suggest the importance of vacuolar systems for polyP biosynthesis in *S. cerevisiae*. There are many indirect data suggesting the difference in metabolic pathways and cellular localization of long-chained and short-chained polyPs in yeasts (Kulaev et al. 2004). For example, short-chained polyPs are localized presumably in cytoplasm, while the long-chained polymers are localized in other organelles and cellular compartments (Lichko et al. 2006). In this study, we demonstrated that the biosynthesis of the shortest chain fraction polyP1 is less dependent on the vacuolar membrane energization. It seems that the biosynthesis of these polyPs involves not only Vtc4, but also other, yet unidentified enzymes.

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