RESEARCH ARTICLE

Effect of a carbon source on polyphosphate accumulation in *Saccharomyces cerevisiae*

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Abstract

The cells of *Saccharomyces cerevisiae* accumulate inorganic polyphosphate (polyP) when reinoculated on a phosphate-containing medium after phosphorus starvation. Total polyP accumulation was similar at cultivation on both glucose and ethanol. Five separate fractions of polyP: acid-soluble fraction polyP1, salt-soluble fraction polyP2, weakly alkali-soluble fraction polyP3, alkali-soluble fraction polyP4, and polyP5, have been obtained from the cells grown on glucose and ethanol under phosphate overplus. The dynamics of polyP fractions depend on a carbon source. The accumulation rates for fractions polyP2 and polyP4 were independent of the carbon source. The accumulation rates of polyP1 and polyP3 were higher on glucose, while fraction polyP5 accumulated faster on ethanol. As to the maximal polyP levels, they were independent of the carbon source for fractions polyP2, polyP3, and polyP4. The maximal level of fraction polyP1 was higher on glucose than on ethanol, but the level of fraction polyP5 was higher on ethanol. It was assumed that accumulation of separate polyP fractions has a metabolic interrelation with different energy-providing pathways. The polyphosphate nature of fraction polyP5 was demonstrated for the first time by ^{31}P nuclear magnetic resonance spectroscopy, enzymatic assay, and electrophoresis.

Introduction

Inorganic polyphosphate (polyP), a linear polymer of many phosphate residues linked by high-energy phosphoanhydride bonds, performs varied functions in microorganisms including yeast, such as phosphate and energy reservation, regulation of enzyme activities, formation of membrane channels, sequestration and storage of cations, and gene activity control (Kulaev & Vagabov, 1983; Reusch, 1992; Kornberg, 1995; Docampo & Moreno, 2001; Kulaev *et al.*, 2004).

Yeast cells are characterized by high level and multiple localization of polyP (Kulaev & Vagabov, 1983; Lichko *et al.*, 2006). Chemical fractionation of polyP from the cells of *Saccharomyces cerevisiae* makes it possible to obtain five separate fractions of these biopolymers: acid-soluble fraction polyP1, salt-soluble fraction polyP2, weakly alkali-soluble fraction polyP3, alkali-soluble fraction polyP4 and, finally, fraction polyP5, which is assessed by the amount of P_i appearing after the hydrolysis of residual material in 0.5 N $HClO_4$ at 90 °C (Vagabov *et al.*, 2000). The synthesis and degradation of these fractions are closely related to metabolic processes in individual cell compartments, and their dynamics is affected in different ways by changing the culture conditions. For example, there are correlations between the synthesis of polyP4 and cell envelope mannoproteins mediated by dolichyl-diphosphate:polyphosphate phosphotransferase (Kulaev *et al.*, 1987) and between RNA synthesis and polyP2 fraction accumulation (Kulaev & Vagabov, 1983).

Because polyP is a compound with high-energy bonds, their metabolism depends considerably on the energy-converting processes in cells. Recently, 255 genes were found to be involved in the maintenance of polyP content in the cells of *S. cerevisiae* (Freimoser *et al.*, 2006). These data confirm the idea of the participation of polyP in cellular energy homeostasis.

Under growth on glucose, even under high aeration, the main mechanism of supplying energy for all metabolic processes in *S. cerevisiae* cells is glycolysis, while respiration and oxidative phosphorylation provide energy under growth on nonfermentable substrates such as ethanol (De

Deken, 1966; Pronk *et al.*, 1996; Merico *et al.*, 2007). These physiological peculiarities make *S. cerevisiae* a good model for studying the effects of energy metabolism on polyP accumulation and usage.

Under short-time cultivation on ethanol, the cells of *S. cerevisiae* accumulated less acid-insoluble polyP than under cultivation on glucose (Schuddemat *et al.*, 1989). In both cases, low-molecular polymers were synthesized first and high-molecular polyP appeared later. However, the effects of a carbon source on accumulation of all the diversities of polyP fractions in *S. cerevisiae* cells are still unknown. The goal of this study is to compare the peculiarities of synthesis of different polyP fractions in *S. cerevisiae* under growth on glucose and ethanol.

Materials and methods

Yeast strain and culture conditions

The yeast *S. cerevisiae* strain VKM Y-1173 was grown in a shaker (200 r.p.m.) in Reader medium containing 2% glucose or 1% ethanol as carbon sources and other components (Vagabov *et al.*, 2000). In phosphate-free medium, potassium phosphate was replaced by KCl and yeast extract by inositol (2 mg L^{-1}), respectively.

The yeast was first cultivated to the early logarithmic growth stage in a complete medium containing 9 mM P_i. Then the cells were harvested, placed in phosphate-free medium, and grown for 7 h. Later, the cells were again placed on fresh complete medium, where the culture was grown for 3.5 h. During the cultivation, biomass samples were harvested at 3000 ***g*** for 10 min, washed twice by distilled water at 4 °C, and used for polyP extraction. Dry cell mass was determined after drying of cell aliquots at 85 °C under vacuum.

Extraction and assay of polyP fractions

PolyP were extracted and assayed as described (Vagabov *et al.*, 2000). On the basis of our previous studies (Vagabov *et al.*, 1998; Kulaev *et al.*, 1999), we consider this extraction method to be one of the best available for separation and quantitative determination of polyP fractions from the yeast cells. PolyP was most completely extracted by this method (Vagabov *et al.*, 1998; Kulaev *et al.*, 1999). Acid-soluble polyphosphates polyP1 were extracted by 0.5 N $HClO_4$ at 0 °C. Salt-soluble polyphosphates polyP2 were extracted by a saturated solution of $NaClO_4$ at 0 °C. Two fractions of alkali-soluble polyphosphates (polyP3 and polyP4) were extracted at 0 °C with weak NaOH (pH of the solution was adjusted to 9–10) and 0.05 M NaOH (pH 12), respectively. The polyP level in the fractions was estimated as a difference in P_i amount before and after the hydrolysis of samples in 1 N HCl for 10 min at 100 °C (Vagabov *et al.*, 2000). The level of polyP5 fraction was determined by the treatment of residual material with 0.5 N $HClO_4$ at 90 °C twice for 20 min, and the assay of released P_i. P_i was determined according to Weil-Malherbe & Green (1951). The data in the tables and figures are average values of three experiments.

^{31}P nuclear magnetic resonance (NMR) spectroscopy of polyP5 fraction

After removal of polyP1, polyP2, polyP3, and polyP4 fractions, residual material was suspended in distilled water, neutralized with 0.1 N HCl, and EDTA was added to a final concentration of 30 mM.

^{31}P-NMR spectra were recorded by an Avance 600 spectrometer (242.9 MHz, 45° pulse, and 1 s delay) in 5-mm standard NMR ampoules. The scan number was 500. The solution of disodium salt of ethylenediaminephosphonic acid with a chemical shift of 12.8 p.p.m. relative to 85% H_3PO_4 was used as a standard. The signal of -21.61 p.p.m. is typical of core phosphate groups of polyP (Vagabov *et al.*, 1998).

Enzymatic assay of polyP

After removal of polyP1, polyP2, polyP3, and polyP4 fractions, residual material was suspended in distilled water, neutralized with 0.1 N HCl, and used for enzymatic assay. The reaction mixture contained 1 mL of 50 mM Tris-HCl, pH 7.2, 2.5 mM $MgSO_4$, 0.1 mL of the above suspension, and 0.1 mL of exopolyphosphatase preparation (1 E mL^{-1}). After incubation at 30° for 30–40 min, the level of polyP was estimated by the amount of P_i released. Pure yeast exopolyphosphatase (Andreeva *et al.*, 2006) was kindly provided by Dr N.A. Andreeva (IBPM RAS).

Electrophoresis of polyP

Fractions polyP1, polyP2, polyP3, and polyP4 were subjected to electrophoresis in 20% polyacrylamide gel with 7 M urea. As for fraction polyP5, after removal of polyP1, polyP2, polyP3, and polyP4 fractions, residual material was suspended in distilled water, neutralized with 0.1 N HCl, and, after centrifugation at 3000 ***g*** for 10 min, the supernatant was subjected to electrophoresis. The gels were stained with 0.05% toluidine blue, 25% methanol, and 1% glycerol, followed by destaining in 25% methanol and 5% glycerol (Pepin & Wood, 1986).

Results and discussion

PolyP accumulation after phosphate starvation

The reinoculation of yeast cells after phosphate starvation to a complete P_i medium resulted in the accumulation of polyP

to a level several times higher than in the cells grown on complete medium without P_i starvation. This accumulation is known as 'hypercompensation' or 'phosphate overplus' (Liss & Langen, 1962; Kulaev & Vagabov, 1983; Kulaev *et al.*, 2004).

The yeast *S. cerevisiae* was grown in the media with glucose or ethanol to the beginning of logarithmic growth phase and then the cells were reinoculated in the media without P_i, and cultivated for 7 h (Fig. 1a). In spite of P_i absence in the media, the biomass amount increased four- and threefold in the media with glucose and ethanol, respectively (Fig. 1a). During the growth in P_i-free media, yeast cells used polyP as an intracellular phosphate source (Fig. 1b). The decrease of the total polyP content at P_i starvation was independent of the carbon source used. As for separate fractions, their content also decreased independent of the carbon source used.

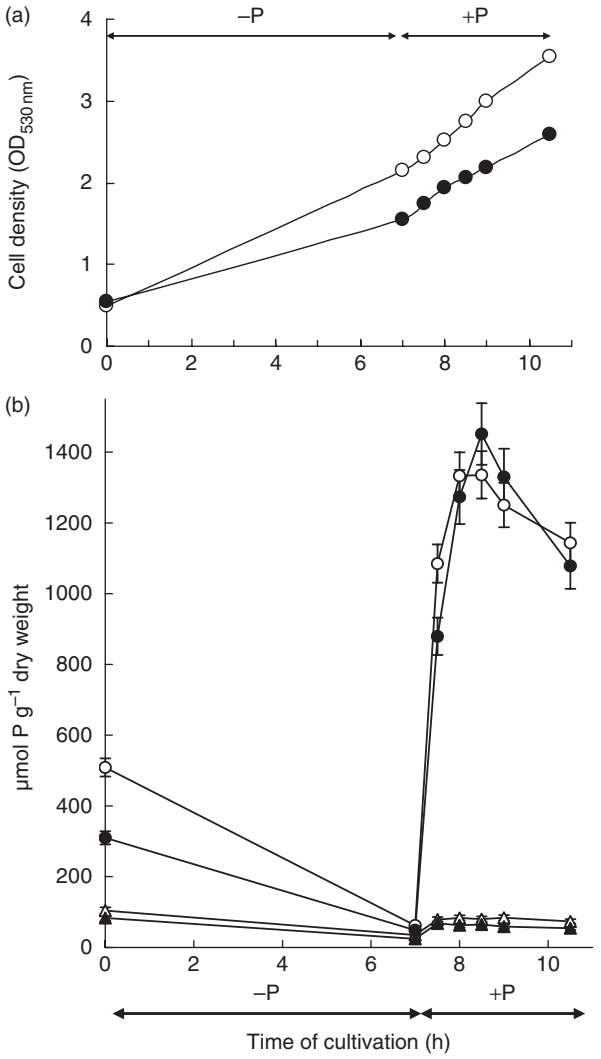

Fig. 1. (a) Growth of *Saccharomyces cerevisiae* VKM Y-1173 in a medium with 2% glucose (○) or 1% ethanol (●), 0–7 h, phosphate starvation; 7–10.5 h, phosphate overplus. The mean values of three determinations with SD of 5% are shown. (b) PolyP and P_i content in the cells of *S. cerevisiae* on phosphate starvation (0–7 h) and phosphate overplus (7–10.5 h): (○) total polyP content under growth on glucose, (●) total polyP content under growth on ethanol, (Δ) P_i content under growth on glucose, (▲) P_i content under growth on ethanol. The mean values of three determinations are shown.

Then P_i-starved cells were reinoculated into complete media. The growth curves are shown in the Fig. 1a. This reinoculation resulted in polyP accumulation while P_i levels changed little (Fig. 1b). This suggests that in order to maintain a fairly constant level of P_i in the cells, the P_i excess was converted into polyP. Yet, after 0.5 h of cultivation in P_i-containing medium with glucose, the total level of polyP increased more than twofold as compared with control (0 h, Fig. 1b) and polyP accumulation continued for 1.5 h (Fig. 1b). Then the polyP level decreased. After 0.5 h of cultivation in P_i-containing medium with ethanol, the total level of polyP increased by nearly threefold as compared with control (0 h, Fig. 1b) and reached the maximum after 1.5 h of cultivation. Then the polyP level decreased as well. As a whole, the total polyP content during phosphate starvation and phosphate overplus change in a similar manner under growth on glucose and ethanol.

Figure 2 shows the dynamics of polyP accumulation in different fractions on growth on glucose and ethanol under phosphate overplus. The rates of accumulation for the first 0.5 h of cultivation in complete medium for fractions polyP2 and to a lesser extent polyP4 were independent of the carbon source. Fractions polyP1 and polyP3 exhibit the maximal accumulation rate at cultivation on glucose, while fraction polyP5 exhibit the maximal accumulation rate on cultivation on ethanol (Fig. 2).

The maximal levels of fractions polyP2, polyP3, and polyP4 did not depend on the carbon source used (Fig. 2). The maximal level of fraction polyP1 was higher on glucose than on ethanol, but that of fraction polyP5 was higher on ethanol.

PolyP3 and polyP5 were the most metabolically active fractions on both ethanol and glucose. Their levels decreased after 1–1.5 h of growth under phosphate overplus (Fig. 2). Thus, these fractions are most likely an energy source under growth in P_i-containing medium.

It is not unlikely that the accumulation of separate polyP fractions is provided by different energy sources. We suppose that the accumulation of polyP1 was probably mediated mainly by glycolysis, while that of polyP5 was mediated mainly by oxidative phosphorylation.

The electrophoresis in polyacrylamide gel electrophoresis (PAGE) demonstrates the difference in chain length of

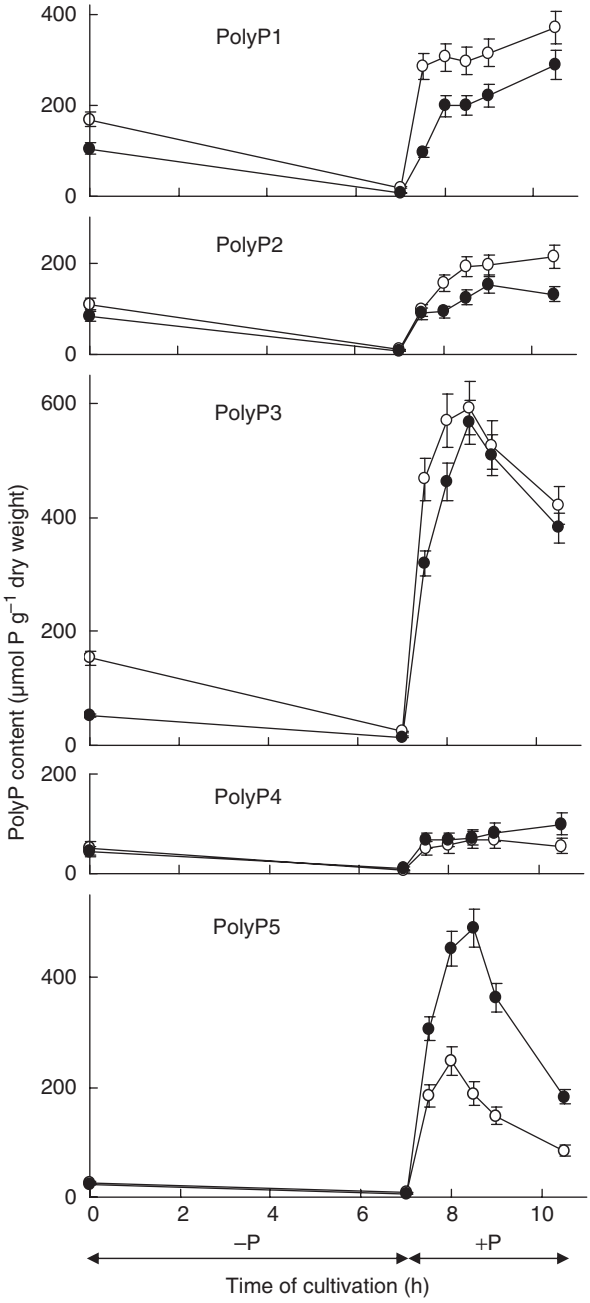

Fig. 2. Content of separate polyP fractions in the cells of *Saccharomyces cerevisiae* on phosphate starvation (0–7 h) and phosphate overplus (7–10.5 h). The culture media contained glucose (○) or ethanol (●). The mean values of three determinations are shown.

separate polyP fractions (Fig. 3). Earlier, similar data were obtained by ^{31}P-NMR spectroscopy during the course of growth in a medium with glucose (Vagabov *et al.*, 2000). The average chain lengths of polyP of different fractions were not affected by the carbon source used under culture conditions (Fig. 3).

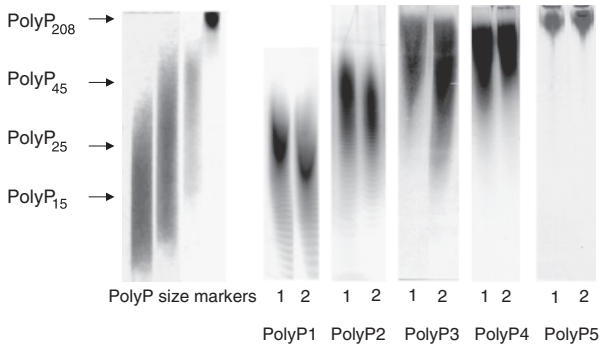

Fig. 3. Electrophoregram of polyP in 20% polyacrylamide gel in the presence of 7 M urea. PolyP standards with an average chain length of 15–208 phosphate residues were used (Sigma); polyP fractions were obtained from the cells *Saccharomyces cerevisiae* grown under phosphate overplus conditions for 0.5 h on glucose (1) and ethanol (2). PolyP5 fraction was obtained as described in Materials and methods.

Evidences of polyphosphate nature of polyP5 fraction

Previously, the level of polyP5 was estimated by P_i amount through the treatment of residual material after the extraction of polyP1–polyP4 by 0.5 N $HClO_4$ at 90 °C. There was no proper evidence of the polyP nature of this fraction, because its content was rather low (5–7% of total polyP in the cells) (Vagabov *et al.*, 2000). At growth on ethanol and under phosphate overplus, the content of polyP5 increased to 30% of total polyP.

In this study, we have demonstrated for the first time that the phosphorus compounds of fraction polyP5 are long-chain inorganic polyphosphates, using ^{31}P-NMR spectroscopy, electrophoresis, and enzymatic assay. For these assays, the material remaining after the extraction of polyP1–polyP4 was treated as described in Materials and methods. Figure 3 demonstrates the electrophoregram of polyP5 fraction in 20% PAGE. Its chain length was maximal among the polyP fractions under study. Figure 4 shows ^{31}P-NMR spectrum of polyP5, which corresponds to the high-molecular polyP signal at 21.6 p.p.m. (Vagabov *et al.*, 1998; Kulaev *et al.*, 2004) P_i was released as a result of treatment of residual material by pure high-molecular cytosol exopolyphosphatase, which is specific to long-chain polyP (Andreeva *et al.*, 2006). If the time of treatment was long enough, all the labile phosphorus of this fraction was converted into P_i. PolyP was not observed under electrophoresis after this treatment. Thus, a strong evidence of the polyP nature of fraction polyP5 has been obtained.

The pathways of polyP biosynthesis in yeasts require further investigations. Considerable synthesis of polyP from ATP was shown in *Candida humicola* (McGrath *et al.*, 2005) while in *S. cerevisiae* the reverse reaction prevailed (Shabalin

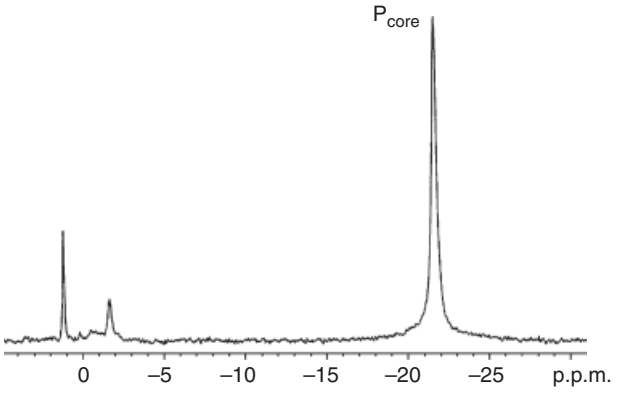

Fig. 4. ^{31}P nuclear magnetic resonance spectrum of the polyP5 fraction obtained from *S. cerevisiae* cells as described in Electrophoresis of polyP. P_{core} indicates the resonance signal of inner phosphate groups of long-chain polyP.

et al., 1977). No gene encoding polyphosphate kinase was found in the *S. cerevisiae* genome (Zhang *et al.*, 2002). The participation of 1,3-diphosphoglucerate polyphosphate phosphotransferase, an enzyme using substrate phosphorylation for polyP and not ATP synthesis, and dolychil polyphosphate kinase in polyP accumulation in *S. cerevisiae* cell is still unclear (Kulaev *et al.*, 2004). Some indirect evidences assume that electrochemical proton gradients on cell membranes support polyP accumulation (Trilisenko *et al.*, 2003; Pestov *et al.*, 2004). The carbon source effect that we observed led us to postulate that the accumulation of different polyP fractions in *S. cerevisiae* cells has a metabolic interrelation with different energy-providing pathways.

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