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ACCELERATED PUBLICATION

Inactivation of *PPX1* and *PPN1* Genes Encoding Exopolyphosphatases of *Saccharomyces cerevisiae* Does not Prevent Utilization of Polyphosphates as Phosphate Reserve

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Abstract—Cytosol polyphosphates (polyPs) are the main phosphate (P_i) reserve in the yeast *Saccharomyces cerevisiae*. In this work, the participation of cytosol polyPs and exopolyphosphatases in maintenance of P_i homeostasis under P_i deficit in the cultivation medium has been studied in different strains of *S. cerevisiae*. The growth of yeast strains with inactivated genes *PPX1* and *PPN1* encoding the yeast exopolyphosphatases and a strain with double mutations in these genes in a P_i-deficient medium is not disturbed. All the studied strains are able to maintain relatively constant P_i levels in the cytosol. In P_i-deficient medium, polyP hydrolysis in the cytosol of the parent and *PPN1*-deficient strains seems to be performed by exopolyphosphatase Ppx1 and proceeds without any change of the spectrum of polyP chain lengths. In the *PPX1*-deficient strain, long-chain polyPs are depleted first, and only then short-chain polyPs are hydrolyzed. In the double *PPX1* and *PPN1* mutant having low exopolyphosphatase activity, polyP hydrolysis in the cytosol starts with a notable delay, and about 20% of short-chain polyPs still remain after the polyP hydrolysis in other strains has almost been completed. This fact suggests that *S. cerevisiae* possesses a system, which makes it possible to compensate for inactivation of the *PPX1* and *PPN1* genes encoding exopolyphosphatases of the yeast cells.

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Inorganic polyphosphates (polyPs) are involved in many functions of microbial cells [1-4] including phosphate (P_i) storage in osmotically neutral form. Limitation of P_i in the growth medium has been studied in the yeast for a long time [5-7]. After prolonged P_i limitation, the yeast is shown to utilize almost the complete polyP pool to support its vitality [8]. There is increasing interest in the study of mechanisms of P_i homeostasis in yeast cells [8-10]. However, it is still unclear what enzymes are involved in the consumption of polyP reserve in the yeast.

At present, only two genes encoding polyP-metabolizing enzymes are identified in *Saccharomyces cerevisiae*: *PPX1* [11] and *PPN1* [12, 13]. *PPX1* encodes exopolyphosphatase splitting out P_i from the end of the polyP chain [11]. The enzyme encoded by *PPN1* was first regarded as endopolyphosphatase splitting long polyP chains to shorter ones [12, 13]. However, this enzyme was shown to have an exopolyphosphatase activity as well [14-16]. The enzyme encoded by the *PPX1* gene more effectively hydrolyzes short-chain polyPs, including $polyP_3$, while the enzyme encoded by *PPN1* preferentially hydrolyzes long-chain polyPs [16, 17].

The 45-kD exopolyphosphatase encoded by the *PPX1* gene is localized in the cytosol, cell envelopes [18], and soluble mitochondrial fraction [17], while the enzymes encoded by the *PPN1* gene and having high molecular masses (120-830 kD) are localized in the nuclei, vacuoles, and mitochondrial membranes of *S. cerevisiae* [19] and appear in the cytosol under *PPX1* inactivation [20] and at early growth stages in P_i-rich medium [16]. The cytosol of yeast cell contains a signifi-

Abbreviations: P_i) phosphate; polyPs) polyphosphates.

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cant amount of polyP and exopolyphosphatases encoded by the *PPX1* and *PPN1* genes depending on cultivation conditions [19].

In this connection, the objective of the present work was to study the effect of *PPX1* and *PPN1* inactivation on polyP utilization in the cytosol of *S. cerevisiae* under P_i limitation in the cultivation medium.

MATERIALS AND METHODS

Strains and culture conditions. Strains of the yeast *S. cerevisiae*—CRY (parent strain), CRX (a strain with inactivated *PPX1* gene), CRN (a strain with inactivated *PPN1* gene), and CNX (a strain with inactivated *PPX1* and *PPN1* genes)—were from the Kornberg's laboratory (Stanford University, USA) [13]. All strains were grown aerobically in a shaker at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) containing 5.7 mM orthophosphate (P_i-rich medium). The media (250 ml) were inoculated directly with the cultures picked from YPD agar slants. The cells were grown for 24 h to the stationary growth phase. Then the cells were separated by centrifugation at 5000*g* for 10 min, washed with sterile distilled water, and re-inoculated into 250 ml of P_i-rich or P_i-deficient YPD medium (2 g wet biomass for each strain). The P_i-deficient medium containing 0.08 mM P_i was prepared as described previously [21]. After cultivation for 1-5 h (exponential growth phase), the cells were centrifuged, washed with distilled water, and used for further analysis.

Preparation and characterization of cytosol. The cytosol fractions were prepared by disruption of spheroplasts in 0.1 M sorbitol followed by centrifugation at 100,000*g* for 3 h as described earlier [22]. The cytosol preparations had no activity of ATPases, which were sensitive to orthovanadate (inhibitor of plasma membrane ATPase), azide and oligomycin (inhibitors of mitochondrial ATPase), and nitrate (inhibitor of vacuolar ATPase), so they were considered free from contamination with these organelles.

The cytosol fractions were subjected to gel filtration on an FPLC Superose 6 column (Pharmacia, Sweden). The elution buffer was 20 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100, 2 mM $MgSO_4$, and 100 mM NaCl. The molecular mass marker kit (Pharmacia) included thyroglobulin (669 kD), ferritin (440 kD), β-amylase (200 kD), aldolase (160 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and cytochrome *c* (13 kD).

Other methods. Preparation of spheroplasts, assay of phosphohydrolase activities, and extraction, assay, and electrophoresis of acid-soluble polyP have been described in detail in our previous paper [23]. The cytosol fractions for polyP assays were obtained in the presence of known exopolyphosphatase inhibitors: 4 mg/ml heparin and 20 mM EDTA [17]. Then equal volumes of 1 N $HClO_4$ were added to the fractions and suspensions were centrifuged at 5000*g* for 20 min. The cytosol fractions for exopolyphosphatase assays were obtained without addition of heparin and EDTA. All experiments have been performed at least three times, and the mean values are presented.

RESULTS

No difference in the growth of all yeast strains was observed in P_i-deficient medium. Growth rates and biomass amounts were the same as in case of P_i-rich medium (not illustrated).

Exopolyphosphatase activities in the cytosol preparations of all strains were assayed after 5-h growth (exponential growth phase), both in P_i-rich and P_i-deficient medium (table). The highest activities were registered in cytosol of the parent CRY strain and in the *PPN1*-deficient mutant CRN in both cases. In P_i-deficient medium, this activity was half as high in the *PPX1*-deficient strain CRX and insignificant in the double mutant CNX (table). Except for the parent strain CRY, the levels of exopolyphosphatase activities detected in P_i-rich media were close to those found under P_i deficit. As for the CRY strain, it had lower exopolyphosphatase activity in P_i-deficient medium as compared with that in P_i-rich medium.

Figure 1 demonstrates the data on gel filtration of the cytosol preparations under P_i deficit. In the cytosol of *PPN1*-defficient strain CRN, exopolyphosphatase activity was represented by Ppx1 of ~45 kD. Just the same was observed for this strain in P_i-rich medium [19]. In the CRY cytosol, a minor amount of ~450 kD exopolyphosphatase activity was present in addition to Ppx1 (Fig. 1).

Exopolyphosphatase activities (mU/mg protein) in cytosol preparations of *S. cerevisiae* in exponential growth phase (5 h)

Strain	Growth conditions	
	P_i-rich medium	P_i-deficient medium
Parent strain CRY	180 ± 7	100 ± 10
Strain CRX with inactivated *PPX1* gene	40 ± 2	55 ± 2
Strain CRN with inactivated *PPN1* gene	70 ± 5	95 ± 5
Strain CNX with inactivated *PPX1* and *PPN1* genes	10 ± 1	6 ± 0.5

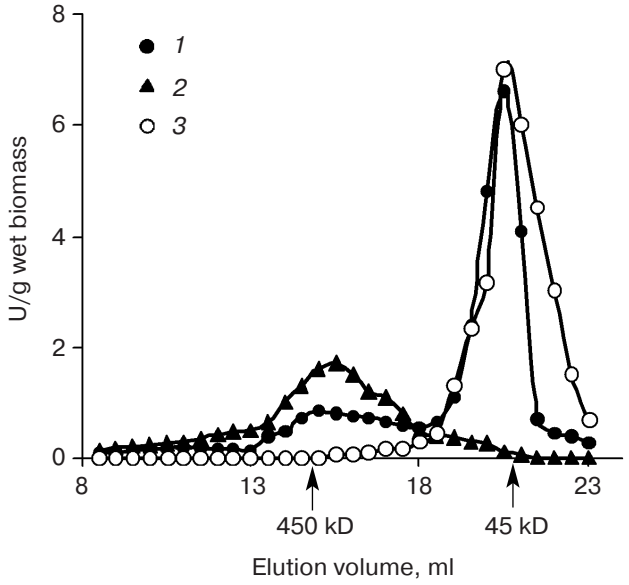

Fig. 1. Exopolyphosphatase activities in Superose 6 gel filtration fractions of the cytosol preparations from *S. cerevisiae*. The cytosols were isolated from cells grown to exponential phase (5 h) in P_i-deficient medium. *1*) Parent strain CRY; *2*) *PPX1*-deficient strain CRX; *3*) *PPN1*-deficient strain CRN.

The contribution of this enzyme to the total exopolyphosphatase activity was half that observed in P_i-rich medium [20]. Only exopolyphosphatase of ~450 kD was revealed in the cytosol of CRX, and Ppx1 was missing in the *PPX1*-deficient strain CRX both in P_i-deficient (Fig. 1) and P_i-rich media [20]. Minor exopolyphosphatase activity in the cytosol preparation of the double mutant CNX (table) was eluted in the region of >1000 kD (not illustrated).

Thus, at exponential growth of the yeast in P_i-deficient medium, exopolyphosphatase activities in the cytosols of strains CRY and CRN were represented mainly by the enzyme Ppx1. Ppn1 was responsible for this activity in the CRX strain.

The polyP levels in the cytosol preparations of all cultures decreased on growth in P_i-deficient medium with rather constant P_i levels (Fig. 2). However, the polyP levels did not decrease similarly in strains with different enzymes in the cytosol. The dynamics of polyP levels in the CRY and CRN strains having the same enzyme Ppx1 was actually similar (Fig. 2). The spectrum of chain lengths was constant in time and similar for strains CRY and CRN (Fig. 3). This fact is consistent with data on substrate specificity of Ppx1 hydrolyzing polyPs with chain lengths of 9-200 phosphate residues at equal rates [22].

In the CRX strain, the rate of polyP hydrolysis was somewhat lower (Fig. 2) with accumulation of short-chain polyPs (Fig. 3) that were not so effectively hydrolyzed by Ppn1 as long-chain ones [16]. However, after long-chain polyPs had been depleted, short polymers were hydrolyzed as well. As a result, the 5-h growth in P_i-deficient medium was followed by polyP depletion in the cytosol preparations of the CRY, CRX, and CRN strains (Fig. 2). In contrast to these strains, about 20% of short-chain polyPs still remained in the cytosol of *PPX1*- and *PPN1*-deficient strain CNX after 5 h of growth (Fig. 2). In this strain, the polyP level moderately decreased within the first 2 h (Fig. 2), whereas the polyP chains were shortening (Fig. 3). Conceivably, fragmentation of long-chain polyPs could occur in this period due to the presence of a certain enzyme possessing endopolyphosphatase activity.

The polyP levels in the cytosol preparations of all cultures decreased not only under growth in P_i-deficient medium, but also under incubation of the preparations at 30°C (Fig. 4). In the CRY and CRN strains having the same enzyme Ppx1 in the cytosol, a sharp decrease of polyPs was observed in the very first minutes of incuba-

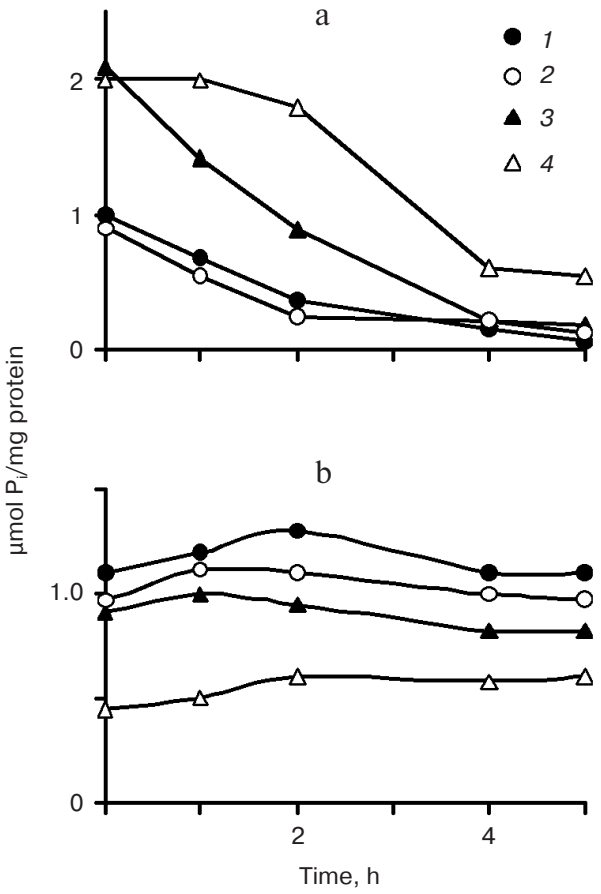

Fig. 2. PolyP (a) and P_i dynamics (b) in cytosol preparations of *S. cerevisiae* under exponential growth in P_i-deficient medium. *1*) Parent strain CRY; *2*) *PPX1*-deficient strain CRX; *3*) *PPN1*-deficient strain CRN; *4*) *PPX1*- and *PPN1*-deficient strain CNX. On isolation of cytosol preparations spheroplasts were lysed in the presence of heparin (4 mg/ml) and 20 mM EDTA, known inhibitors of exopolyphosphatases [17].

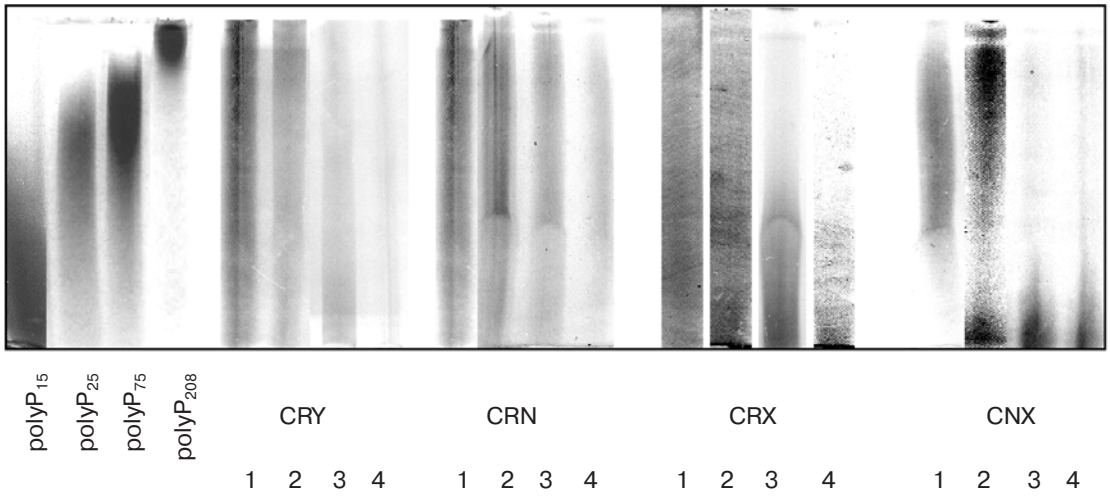

Fig. 3. Electrophoresis of cytosol polyPs in 20% polyacrylamide gel. Chain lengths of polyP markers are indicated on the left. The numbers below indicate hours of growth of the strains in P_i-deficient medium.

tion. PolyP hydrolysis in the CRX cytosol (Ppn1 enzyme) was slower and completed only in 120 min (Fig. 4). The polyP level in the cytosol of the double mutant CNX did not change within the first hour of incubation and decreased by ~40% in 4 h (Fig. 4).

DISCUSSION

The results suggest that the cytosol polyPs serve as P_i reserve and are effectively used under P_i limitation in the cultivation medium. Deficit of P_i in the cultivation medium has but a minor effect on exopolyphosphatase activities in the cytosol of all studied strains as compared with the same growth phase in P_i-rich medium (table), i.e. enzymes encoded by the *PPX1* and *PPN1* genes are not induced under P_i deficit. In this respect, the yeast differs from bacteria. Limitation of P_i in *Escherichia coli* and *Acinetobacter johnsonii* is known to induce expression of the *ppx* gene, followed by a drastic increase of exopolyphosphatase activity [24, 25].

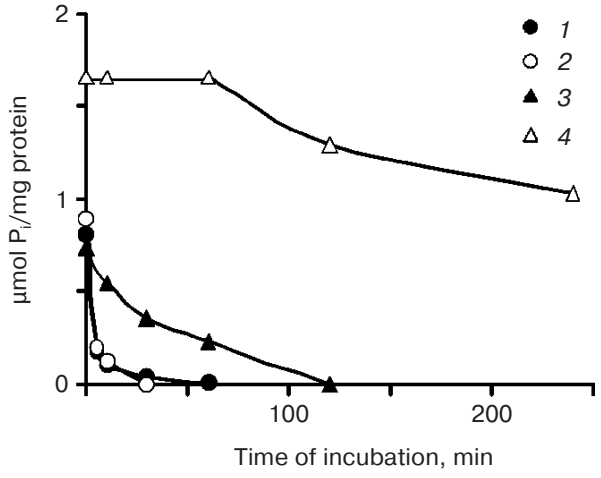

Fig. 4. Polyphosphate dynamics in cytosol preparations of *S. cerevisiae* isolated from cells grown to stationary growth phase in P_i-rich medium under their incubation at 30°C: *1*) parent strain CRY; *2*) *PPX1*-deficient strain CRX; *3*) *PPN1*-deficient strain CRN; *4*) *PPX1*- and *PPN1*-deficient strain CNX. On isolation of cytosol preparations spheroplasts were lysed in the absence of inhibitors of exopolyphosphatases—heparin and EDTA.

As regards the cytosol of *S. cerevisiae*, the CRY and CRN strains possess mainly exopolyphosphatase Ppx1, which is the enzyme that hydrolyzes the cytosol polyPs as confirmed by the dynamics of chain-length changes. Exopolyphosphatase Ppn1 performs this function in the *PPX1*-deficient strain CRX. This enzyme hydrolyzes first of all the long-chain polyPs and then the short-chain polyPs, to which it has less affinity [16]. In the double mutant CNX lacking both enzymes, polyP hydrolysis proceeds with a notable delay but does not affect culture growth both in P_i-deficit and P_i-rich media. Exopolyphosphatase activity in the cytosol of this strain is insignificant, and polyP degradation is supposed to be performed by alternative enzymes. The problem of availability of polyphosphate kinase in *S. cerevisiae*, which is able to catalyze both polyP synthesis and degradation, still has to be solved, though this activity has been found recently in the yeast *Candida humicola* [26]. The question of the presence of endopolyphosphatase in *S. cerevisiae* is still unclear. In the double mutant, polyP degradation is followed by accumulation of short-chain polyPs, which might be hydrolyzed by a pyrophosphatase [27]. Such dynamics of polyP hydrolysis suggests the occurrence of endopolyphosphatase not encoded by the *PPN1* gene.

The findings suggest that *S. cerevisiae* possesses a system that makes it possible to compensate for the inactivation of *PPX1*, *PPN1*, or both of these genes encoding exopolyphosphatases in the cytosol.

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