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In vitro detection and characterisation of a polyphosphate synthesising activity in the yeast *Candida humicola* G-1

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Abstract

An in vitro detectable polyphosphate-synthesising activity was characterised using two independent assay systems in extracts of the yeast *Candida humicola* G-1. Its properties were similar to those of a range of bacterial polyphosphate kinase enzymes. PCR amplification of *C. humicola* genomic DNA using universal primers for bacterial polyphosphate kinase genes yielded a product whose translated sequence showed up to 34% amino acid similarity to the bacterial enzyme.

Keywords: Polyphosphate; Yeast

1. Introduction

The bacterial metabolism of the biological polymer of phosphate (P), polyphosphate (polyP) involves two main enzymes. Polyphosphate kinase (polyphosphate: ADP phosphotransferase; PPK; EC 2.7.4.1) catalyses the reversible transfer of the terminal P of ATP to polyP while exopolyphosphatase (PPX; EC 3.6.1.11) sequentially hydrolyses the terminal P residues of the polyP chain. Both the biochemistry and genetics of bacterial PPK and PPX have been extensively studied with the respective enzymes purified and their genes cloned and sequenced (reviewed in [10, 13]). However, those enzymes involved in eukaryotic polyP metabolism have remained more elusive even though the identification of polyP in yeast cells was first reported in 1888 [17].

In cells of *Saccharomyces cerevisiae* polyP accumulation can exceed 23% of the cellular dry weight [20]. This would suggest a central role in cell physiology although the exact biological function of polyP remains unresolved (reviewed in [10,13]. Exopolyphosphatase activities have been detected in cells of *S. cerevisiae* with the gene encoding a cytosolic PPX (scPPX1) described [36,37]. Two further *S. cerevisiae* exopolyphosphatases (scPPX2 and scPPX3), and an endopolyphosphatse activity (Ppn1) have also been identified [14,16,30,37]. In contrast, *S. cerevisiae* genomic expression analysis has failed to detect a *ppk* gene, while no in vitro demonstration of a polyP synthesising activity within yeasts exists [11,24]. This is puzzling especially given their anticipated role within the enhanced biological phosphate removal process and the intracellular abundance of the polyP biopolymer [20,29]. Using whole genome DNA microarray analysis, under conditions of P starvation, four genes (*PHM1, PHM2, PHM3,* and *PHM4*) have been shown to play a role in the intracellular metabolism of polyP by *S. cerevisiae*. Mutations in these results in severely deficient polyP accumulation under conditions of 'polyphosphate overplus', i.e., a period of P starvation followed by incubation in high P media [8,24]. Yet the activities of these gene products have still to be demonstrated in vitro while their involvement in polyP synthesis under standard growth conditions is still unclear. The *PHM* genes also show no ho-

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mology to their functionally equivalent *Escherichia coli ppk* counterparts [4].

Research within our laboratory has recently identified an environmental yeast, *Candida humicola* G-1, in which intracellular polyP accumulation is stimulated during active growth at acid pH under fully aerobic conditions [18]. In this paper we demonstrate, using two independent assays, a polyP synthesising activity in *C. humicola* G-1 cell extracts and present an investigation of its properties.

2. Materials and methods

2.1. Organism, growth conditions and preparation of cell extracts

C. humicola G-1, recently isolated by us from activated sludge, was grown at pH 5.5 using a glucose mineral salts medium as previously described [18]. Cells were harvested, in mid-log phase when polyP accumulation was known to be maximal, at 10 000 *g* for 15 min at 4 °C and washed twice in 1.5 M NaCl. The cell pellet was resuspended in 2 ml wash buffer and disrupted at 40 000 psi using a Constant Systems One-Shot cell disrupter (Constant Systems Ltd., Warwickshire, UK). Cell homogenate was centrifuged at 25 000 *g* and 4 °C for 30 min and the resultant supernatant or crude extract stored at 4 °C for not more than 24 h. Cell-free extracts, prepared from *C. humicola* G-1 cells grown at pH 7.5 when polyP accumulation did not occur, were used as controls.

2.2. Enzyme assays

PolyP synthesis was assayed in vitro by the method of Mullan et al. [22] which directly measures polyP formation, unlike many indirect assays used in other studies. The assay mixture (0.5 ml) consisted of: 50 mM Tris–HCl buffer (pH 7.0), 40 mM glycylglycine-KCl, 10 mM potassium phosphate (pH 7.0), 10 mM $MgCl_2$, 15% (vol/vol) glycerol, 2.5 mM ATP and 5 mM Type 75 PolyP (Sigma). The reaction was initiated by the addition of 25 µl of *C. humicola* G-1 cell extract (0.15–0.25 mg protein) and incubated for 10 min at 20 °C. The net amount of polyP produced was determined as previously described using the metachromatic dye toluidine blue [21]. Activity was expressed as the mean of three measurements, in µmol of polyP produced (as P residues) per min per milligram of cell extract protein. In addition to the method of Mullan et al. [22] polyP synthesising activity in cell extracts of *C. humicola* G-1 was further demonstrated using the well-established PPK assay of Robinson and Wood [28]. This indirectly measures polyP synthesis through the consumption of ATP which is determined via an ATP regeneration reaction using pyruvate kinase, lactate dehydrogenase and NADH. The consumption of the latter is monitored at 340 nm [28].

2.3. PCR amplification

Total DNA from cells of *C. humicola* G-1 was isolated using the DNeasy Tissue kit (Qiagen). The nucleotide primers NLDE-F and TGNY-R, previously designed to amplify 1300-bp internal *ppk* fragments from various PPK-expressing bacteria [19], were used. Amplification (PCR) reactions were conducted with 100 ng of total DNA using Advantage 2 polymerase mix and 0.5 mM GC-melt (Clontech). Reactions were run as follows: initial denaturation at 95 °C for 5 min and then 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min with a final extension for 10 min at 72 °C. The PCR product obtained was purified with a BioRad purification kit and cloned into pGEM-TEasy vector (Promega). The nucleotide sequences of both strands were determined. Initial computer analysis of the sequences was performed using the DNASIS (Hitachi) software package. The alignment of sequences was performed using CLUSTALW [31] with gap open penalty set at 100 and gap extension penalty at –10. Other parameters were set at default values. The deduced amino acid sequence was compared with previously reported PPK sequences: *Bacillus halodurans* C-125 (accession No. 10173727), *Campylobacter coli* (2239078), *E. coli* K12 (1788839), *Helicobacter pylori* J99 (4154939), *Acinetobacter* sp. ADP1 (2462044), *Xylella fastidiosa* (9107809), *ppk* homolog type I (AF502189), *ppk* homolog type II (AF502196), *ppk* homolog type III (AF502194), *ppk* homolog type IV (AF502195) and *Rhodocyclus tenuis* (AF502199). Searches for nucleotide and amino acid sequence similarities were carried out using the FASTA and BLAST programs [26] in the EMBL and GenBank databases.

3. Results

3.1. In vitro detection of polyphosphate synthesising activity

When cell extracts, prepared from *C. humicola* G-1 cells grown to mid-log phase at pH 5.5, were incubated in assay buffer in the presence of Type 75 polyP (0.5 g/L) and 2.5 mM ATP a net increase in polyP concentration was detected by toluidine blue metachromasy. The rate of polyP formation was directly proportional to both the amount of protein present and to the assay time (Figs. 1 and 2). No change in polyP concentration was observed in assays from which cell extract had been omitted.

3.2. Polyphosphate synthesising activity: effects of pH, temperature, metal ions and phosphate

The effect of pH on *C. humicola* G-1 polyP synthesising activity was examined over the pH range 4.0–8.8. The enzyme showed highest activity in the range 7.0–7.5 (Fig. 3).

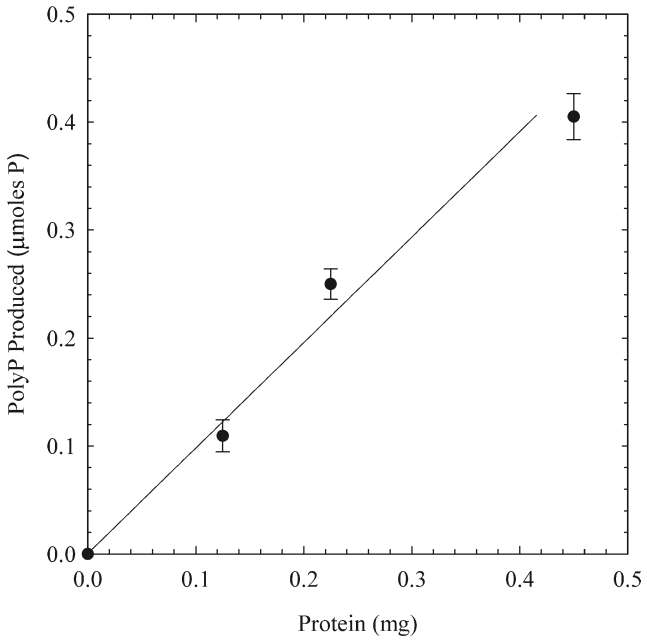

Fig. 1. Polyphosphate synthesis by cell extracts of *C.humicola* G-1 using varying concentrations of protein (0–0.45 mg/ml). Polyphosphate determination was carried out via the toluidine blue assay at 20 °C for 10 min.

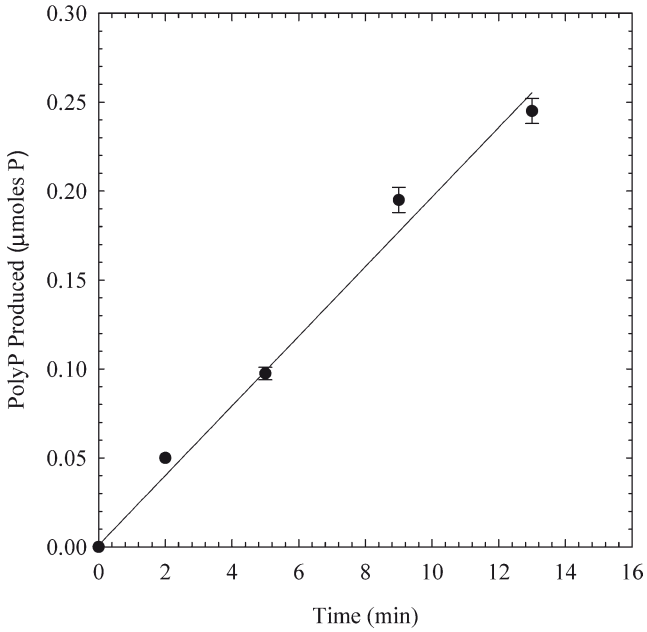

Fig. 2. Polyphosphate synthesis by cell extracts of *C. humicola* G-1 over varying time periods (0–14 min). Polyphosphate determination was carried out via the toluidine blue assay at a protein concentration of 0.225 mg/ml at 20 °C.

The optimum temperature was found to be approximately 37 °C (Fig. 4) and activity was rapidly lost during exposure to temperatures in excess of 40 °C.

Investigation of enzyme activation through the addition of metal ions to untreated cell extracts of *C. humicola* G-1 showed that neither the addition of Mn^{2+} nor

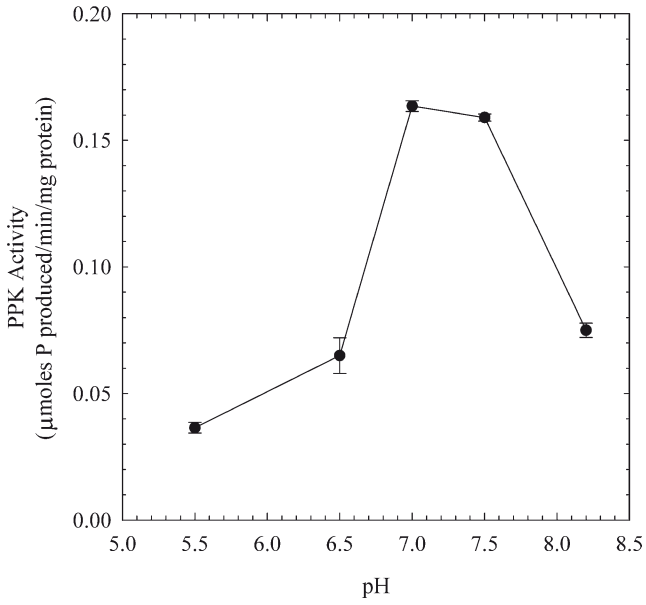

Fig. 3. Effect of pH on the *C. humicola* G-1 polyphosphate synthesising activity.

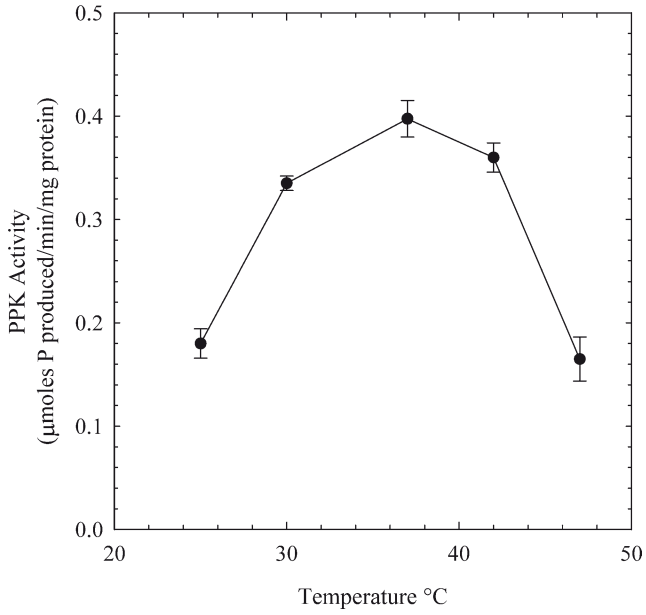

Fig. 4. Effect of temperature on the *C. humicola* G-1 polyphosphate synthesising activity using an incubation time of 10 min.

Zn^{2+} produced any detectable activity. However, the addition of Mg^{2+} (5 mM) resulted in a polyP synthesising activity of 0.11 μmol P/min/mg cell extract protein while Ca^{2+} (5 mM) resulted in a polyP synthesising activity of 0.05 μmol P/min/mg cell extract protein; the greatest levels of activation were obtained in the presence of 10 mM Mg^{2+}. In addition to metal ion activation the in vitro polyP synthesising activity was also dependent upon P concentration. Maximal activity (0.19 μmol P/min/mg of cell extract protein) occurred in the presence of 10 mM P: no detectable PPK activity was observed when P was omitted from the as-

say. PolyP (chain length 75 containing no detectable free P; 5 mM) also stimulated the activity, albeit 10-fold less than in the presence of an equivalent concentration of P.

3.3. Determination of K_m, V_{max} and enzyme specificity

The kinetic constants of the polyP synthesising activity present in cell extract preparations from *C. humicola* G-1 were determined by the toluidine blue assay system [21]. Lineweaver–Burk plots were used to calculate K_m and V_{max}. The enzyme's apparent K_m (in the presence of 10 mM Mg^{2+} and 10 mM potassium phosphate at pH 7.0 and 20 °C) was 5.5 mM ATP with an apparent V_{max} of 1.33 μmol P/min/mg of cell extract protein. The utilisation of ATP for chain elongation was confirmed using the ATP-regenerating method of Robinson and Wood [28]. This assay has previously been successfully used in the determination of bacterial PPK activity [28]. Using this latter approach an apparent K_m of 4.75 mM ATP and a V_{max} of 0.91 μmol P/min/mg of cell extract protein was detected. No in vitro polyP synthesis was observed when ATP was replaced in either assay by GTP, CTP or TTP.

3.4. PCR amplification, analysis of putative product and comparison with prokaryotic PPK sequences

The biochemical characteristics of the polyP synthesising activity in *C. humicola* G-1 cell extracts appear to be strikingly similar to those properties previously described for prokaryotic PPKs. As universal bacterial *ppk* primers have recently been described [19] we decided to utilise these in an attempt to retrieve a homologous sequence from *C. humicola* G-1 DNA. Using these primers, under the PCR conditions described, we obtained a fragment of 1183 nucleotides. The translated amino acid sequence was compared with known bacterial PPK sequences. *C. coli* PPK and *H. pylori* J99 PPK showed the highest levels of amino acid similarity, 32% (14% of residues identical and 18% strongly similar) and 34% (12% of amino acids identical, 22% strongly similar) respectively. Sequence alignments of the *C. humicola* G-1 fragment and *C. coli* PPK are presented in Fig. 5.

4. Discussion

Although the discovery of inorganic polyP in yeast dates back over one hundred years the enzymatic basis of polyP

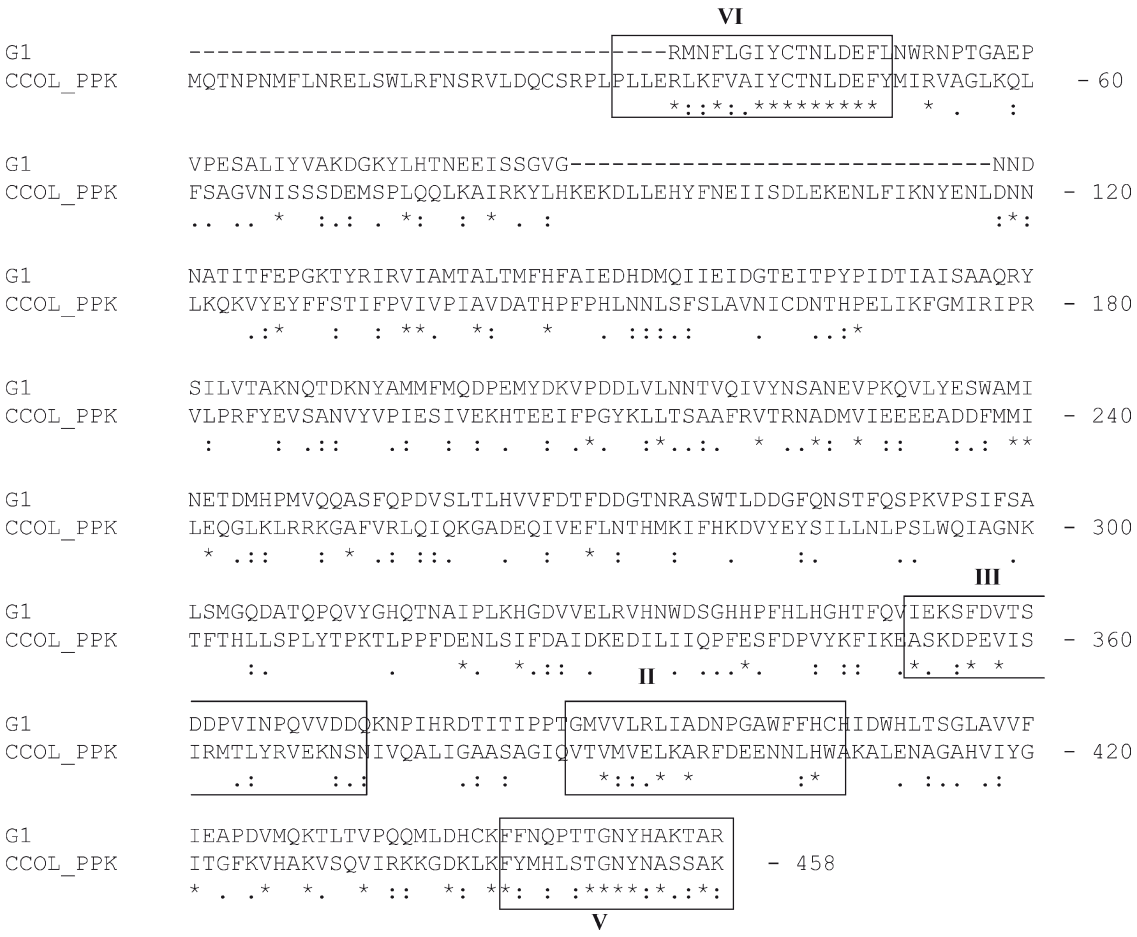

Fig. 5. Alignment of *C. coli* PPK with an amino acid sequence derived from *C. humicola* G-1. Numbers II, III, V and VI refer to regions of high homology previously described from prokaryotic PPK alignments [35].

synthesis in eukaryotes remains unresolved [13,24]. Currently, no in vitro demonstration of a polyP synthesising activity within yeasts exists [24]. We have previously described the isolation of an environmental yeast, *C. humicola* G-1, which accumulates intracellular polyP in response to an acid environment [18]. When cell extract preparations from cells of this isolate, grown at pH 5.5, were incubated in the presence of Type 75 polyP (5 mM) and ATP (2.5 mM) a net increase in polyP concentration could be detected using toluidine blue metachromasy [22] (Figs. 1 and 2). This technique, previously developed by us, is an effective non-radioactive alternative for the in vitro measurement of polyP synthesising activity, with its main advantage being the direct measurement of polyP concentration increase. This is essential as failure to fully quantify polyP production has resulted in the previous misidentification of an in vitro polyP forming activity within *S. cerevisiae* [3,7]. This reported activity was believed to be the first identification of a yeast PPK but, due to an ambiguity in the assay system used, it was actually an exopolyphosphatase working in concert with the ADP-P phosphate exchange activity of the enzyme diadenosine-5′,5‴-P^1,P^4-tetraphosphate α, β-phosphorylase [3]. Our toluidine blue assay, by directly measuring changes in polyP concentration, prevents interference by enzymes such as diadenosine-5′,5‴-P^1,P^4-tetraphosphate α, β-phosphorylase and adenylate kinase. We have previously successful utilised this procedure to investigate PPK activity present in cell extracts of *Burkholderia cepacia* AM19 [22]: this isolate similarly displays enhanced P uptake and polyP accumulation in response to acid stress. Confirmation of in vitro polyP synthesis in *C. humicola* G-1 cell extracts was achieved using the established and widely cited ATP regenerating protocol of Robinson and Wood [28]. This assay indirectly measures PPK activity through the consumption of ATP. Using both methods an unambiguous polyP synthesising activity could be detected in cell extracts of *C. humicola* G-1. Both these assay systems were therefore further exploited to determine the biochemical properties of the activity. This study represents the first in vitro demonstration of enzyme-catalysed polyP formation in yeasts.

The biochemical investigation of *C. humicola* G-1 polyP synthesising activity revealed a striking similarity to previously characterized bacterial PPKs. For example activation of in vitro polyP synthesis by divalent cations and in particular Mg^{2+} is a characteristic common to all prokaryotic PPKs [23,24,32,34]. Kinetically, using the toluidine blue assay, the activity had an apparent K_m of 5.5 mM ATP and V_{max} of 1.33 μmol P/min/mg of cell extract protein while an apparent K_m of 4.75 mM ATP and a V_{max} of 0.91 μmol P/min/mg of cell extract protein was determined using the Robinson and Wood method [28]. These results compare with K_m values for purified PPKs from *E. coli* (2.0 mM ATP) [1], *Neisseria meningitidis* (1.5 mM ATP) [33], *Acinetobacter* sp. strain ADP1 (1.0 mM ATP) [34] and *Vibrio cholera* (0.05 mM ATP) [25] and the PPK of *B. cepacia* AM19 assayed in cell extracts (1.18 mM ATP) [22].

Of particular interest from those studies on prokaryotic PPKs is the apparent stimulation of kinase activity in the presence of P and polyP [33]. The PPK activities of *N. meningitidis*, *Propionibacterium shermanii* and *B. cepacia* AM19 are stimulated 6-fold, 10-fold and 30-fold, respectively, by the addition of P to the reaction buffer [22,27,33]. *C. humicola* G-1 in vitro polyP synthesising activity shows a similar dependence upon P with maximal activity (0.19 μmol P/min/mg of cell extract protein) occurring in the presence of 10 mM P. Stimulation of PPK activity by P is a characteristic of the regulation of this enzyme in many prokaryotes. Indeed, competitive inhibition of the alleged *S. cerevisiae* PPK by P provided further confirmation of its true identity as diadenosine-5′, 5‴-P^1,P^4-tetraphosphate α, β-phosphorylase rather than PPK [3]. This activation may reflect either a requirement of P to act as a primer for polyP chain synthesis or an allosteric effect [3]. Given the potential physiological role of polyP in the maintenance of intracellular P levels, such a fine control mechanism could provide a means by which polyP synthesis would be enhanced in response to high intracellular P concentrations [3] [33]. Activation of polyP synthesis by P would therefore provide one mechanism through which cells could control in vitro activity and intracellular P/polyP concentrations.

The polyP synthesising activity from *C. humicola* G-1 is specific in its utilisation of ATP as the phosphoryl donor for polyP chain elongation with no detectable biosynthetic activity observed in the presence of GTP, CTP or TTP. Specificity for ATP as the nucleoside triphosphate for the formation of polyP has been similarly observed in *E. coli* [15], *N. meningitidis* [33] and *B. cepacia* AM19 PPK [22]. This exclusive use of ATP may suggest a role for polyP in regulating the cellular energy charge [33].

From these biochemical results it would appear that the polyP synthesising activity present in cell extracts of *C. humicola* G-1 has many features in common with those of prokaryotic PPKs. We therefore attempted to retrieve, using PCR, a *ppk* homologue from *C. humicola* G-1. The universal primers NLDE-F and TGNY-R have recently been developed to retrieve fragments of putative *ppk* genes from samples of activated sludge displaying enhanced biological phosphate removal: four novel *ppk* homologues were found in activated sludge biomass using this approach [19]. Amplification with these primers in bacteria gives a 1300-bp fragment [19]. No sequences with significant homology to those encoded by the primers were detected in any available eukaryotic database apart from an internal portion of NLDE-F (nucleotides: 6–26) which is 90% identical to a *Schizosaccharomyces pombe* chromosome 1 sequence. Using these primers we obtained a fragment close to the expected size (1183 nucleotides). The sequence showed highest amino acid similarity—32%—with *C. coli* (14% of residues identical and 18% strongly similar; Fig. 5) and 34% similarity with *H. pylori* J99 (12% of amino acids identical, 22% strongly similar). Prokaryotic PPK sequences contain 10 highly homologous motifs, with 4 of these occurring in the first 460

residues of the amino terminus (Fig. 5) [35]. We also observed stronger conservation within some of the regions. Region VI displayed the highest level of homology with 55% of the amino acids identical, 33.3% strongly similar and 5% weakly similar to *C. coli* PPK (Fig. 5). The amino acids of Region V were 35% identical, 30% strongly similar and 5% weakly similar while the residues of Region 11 were 20% identical, 15% strongly similar and 5% weakly similar. Region 111 had the lowest level of amino acid similarity with 15% identical, 20% strongly similar and 15% weakly similar (Fig. 5). From prokaryotic PPK alignment studies the lowest degree of homology is observed between residues ∼130 and 320 [35]. This may account for our low overall, but still statistically significant, level of amino acid homology despite the clear conservation within the four motifs. The % amino acid similarity between the *C. humicola* G-1 fragment and *C. coli* PPK is therefore consistent with previous alignment studies within prokaryotic PPK sequences [35].

When compared to existing database entries the *C. humicola* G-1 fragment also showed similarity to a number of putative yeast oxidase sequences, with up to 40% of amino acid identity (accession No. 22506642 and 15146339). No significant similarity was observed between the *C. humicola* G-1 fragment and the *PHM1, PHM2, PHM3,* and *PHM4* genes of *S. cerevisiae* (amino acid similarities were in the region 2–6%). These four *PHM* genes, previously shown to be essential for polyP accumulation in *S. cerevisiae* by the 'polyP overplus' phenomenon, are also identical to the *VTC2, VTC3, VTC4* and *VTC1* genes respectively, which are involved in a vacuolar transporter chaperon [5] and function as regulators of vacuolar H^+-ATPase activity [2,5,24]. As genomic expression analysis has failed to detect a *ppk* gene in *S. cerevisiae* it has been suggested that in this yeast P is incorporated into polyP by utilising the proton motive force to drive phosphoanhydride bond formation [24]. No in vitro polyP synthesising activity has, however, been described for these proteins [24].

Taken together, the biochemical results using two independent assay methods, the similarity between the *C. humicola* G-1 fragment and *C. coli* PPK, coupled with the lack of similarity to the *PHM* genes of *S. cerevisiae,* would suggest that, at least in some yeast species, a polyP synthesising activity similar to prokaryotic PPK may exist. This could be a reflection of the conditions under which polyP accumulation occurs in *C. humicola* G-1. PolyP studies on *S. cerevisiae* have traditionally been carried out using the 'polyP overplus' method to induce polyP accumulation. *C. humicola* G-1 accumulates up to 5.7% of its dry weight as P during active growth at pH 5.5 under normal culture conditions: no prior P starvation is required to induce polyP accumulation. By contrast, polyP accumulation by *S. cerevisiae* at acid pH is minimal during the maximal growth phase and increases only during stationary phase [9]. Additionally, the *PHM* genes of *S. cerevisiae* belong to the group of P starvation (PHO-regulated) genes whose expression is triggered by P starvation [24]. Their role in the accumulation of intracellular polyP under high P conditions, as is the case for *C. humicola* G-1, remains unresolved and might suggest the existence of alternative polyP synthesising routes. Indeed the existence of several polyP producing pathways has been described in *E. coli* CA10 [6], *N. meningitidis* BNCV [32], *P. aeruginosa* 8830 [38] and *Acinetobacter* sp. strain ADP1 [34] while the synthesis of yeast surface high molecular weight polyP occurs via a pathway closely resembling that involved in the biosynthesis of cell wall mannoproteins and catalysed by the enzyme dolichylpyrophosphate:polyP phosphotransferase [12].

This report demonstrates, for the first time, an in vitro polyP synthesising activity in a yeast isolate. Biochemical and genetic characterisation suggests that this activity may be similar to that of PPK enzymes of prokaryotes.

Acknowledgements

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