MICROBIAL CELL FACTORIES

REVIEW | Open Access

Polyphosphate - an ancient energy source and active metabolic regulator

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Abstract

There are a several molecules on Earth that effectively store energy within their covalent bonds, and one of these energy-rich molecules is polyphosphate. In microbial cells, polyphosphate granules are synthesised for both energy and phosphate storage and are degraded to produce nucleotide triphosphate or phosphate. Energy released from these energetic carriers is used by the cell for production of all vital molecules such as amino acids, nucleobases, sugars and lipids. Polyphosphate chains directly regulate some processes in the cell and are used as phosphate donors in gene regulation. These two processes, energetic metabolism and regulation, are orchestrated by polyphosphate kinases. Polyphosphate kinases (PPKs) can currently be categorized into three groups (PPK1, PPK2 and PPK3) according their functionality; they can also be divided into three groups according their homology (EcPPK1, PaPPK2 and ScVTC). This review discusses historical information, similarities and differences, biochemical characteristics, roles in stress response regulation and possible applications in the biotechnology industry of these enzymes. At the end of the review, a hypothesis is discussed in view of synthetic biology applications that states polyphosphate and calcium-rich organelles have endosymbiotic origins from ancient protocells that metabolized polyphosphate.

Introduction - polyP origins

The first law of thermodynamics states that energy is neither created nor destroyed but can be converted from one form to another. Biological systems are beautiful models of this law in which the energy transformed into chemical potential energy is stored in covalent bonds between atoms. Later, potential energy, released by breaking certain chemical bonds, is used for biological reactions [1]. Inorganic polyphosphate (polyP) is a rich source of energy. PolyP compounds are linear polymers containing tens to hundreds of phosphate residues linked by energy-rich phosphoanhydride bonds (Figure 1) [2].

PolyP appears to have always been an easy and rich source of energy from prebiotic times to today. Unfortunately, no abiotic polyP minerals can be found on Earth today. However, some calcium pyrophosphate has been found in New Jersey and small amounts of pyrophosphate and tripolyphosphate have been found in fumaroles near Mount Usa in Hokkaido, Japan [3]. PolyP has also been found in other areas, such as the polyP found

in deep oceanic steam that is a biogenic amorphous mineral. Those polyP compounds composed of calcium orthophosphates are produced from the exoskeleton structures of dead plankton [4]. For this reason, some authors think that polyP-like matter is produced only through an organism-mediated process, and so its abiotic origin in marine environments is unlikely [5]. Despite the fact that today's marine polyP has a biotic origin, one would agree with Kornberg's theory that polyP represents a "bioenergy fossil". It is a prominent energy precursor in prebiotic evolution [6] if the following three points are considered:

(i) First, pyrophosphate and polyP are simply produced by heating inorganic phosphate under anhydrous conditions [7]. This is a well-described method used by manufacturers of polyphosphate glass. For example, sodium metaphosphate is manufactured by heating two parts sodium nitrate and one part phosphoric acid. Sodium metaphosphate can be prepared by the dehydration of sodium phosphate. Sodium trimetaphosphate is manufactured by heating and subsequently cooling sodium hexametaphosphate at 500°C for 8 to 12 hours [8]. In light of this, it is easy to see how polyP could be abiotically accumulated at high temperatures under

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Figure 1 The polyphosphate molecule.
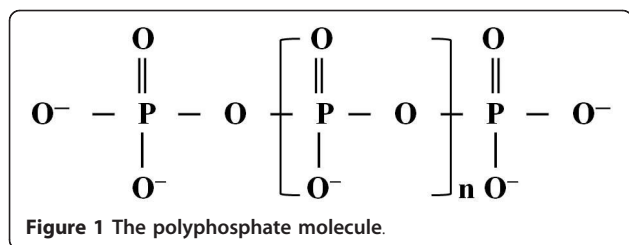
anhydrous conditions during formation of the primitive Earth in which the accretion of material was heated at the core and released as steam into the atmosphere. Similar to phylosilicates, phosphoric acid salts could also bring water to the Earth's surface [9]. Additionally, it was shown that marine volcanic activity could produce water-soluble polyphosphates through partial hydrolysis of longer polyPs [3].

(ii) Second, known polyphosphate kinases (PPKs), enzymes that can mediate the synthesis and degradation of polyP chains [10], are widely distributed in microorganisms. In fact, polyP is found in each type of cell in nature [6,11].

(iii) Third, polyP can help organisms adapt to extreme conditions such as salinity, osmolarity, desiccation, UV radiation, barometric pressure, pH and temperature [12,13]. Such adaptations could have been useful for the first primitive organisms living in the conditions of a primitive Earth [14]. It was reported that *ppk1* mutants lacking polyP are more sensitive to hydrogen peroxide, high temperatures and salt levels as compared to the wild type [15].

PolyP in living cells

PolyP was first found as metachromatic granules in the cytoplasm of the bacterium *Spirillum volutans*, and so it was referred to as "volutin". These particles were stained pink by basic toluidine blue and were later found in other microorganisms [16]. Using electron microscopy, "volutin" granules were seen to be highly refractive and appeared to volatilize while viewed under the electron beam. Correlation between the microscopically observed number of volutin granules and the polyP cell count led to the identification of their main component as polyP. "Volutin" granules were then renamed polyP granules [17]. PolyP has since been found to be present in every cell in nature including bacterial, fungal, plant and animal cells [11].

PolyP granules contain "acid-insoluble" polyP with long-chains [2,18] and are present in the cytoplasm of various prokaryotes [6,11]. In bacterial cells, there is also "acid-soluble" polyP with short-chains [2,18] that can be found in various cell compartments (on the cell surface, in the perisplasm, and in the plasma membrane). In the *Neiseria* species, for example, polyP is capable of

forming capsule-like coatings attached to the cell-surface membrane [19]. In *Helicobacter pylori*, polyP granules are detectable in the cytoplasm in association with the cell membrane; compact polyP particles can be visualized at the flagellar pole [20]. The total cellular pool of polyP depends on the phosphate concentration around the cell. Some bacteria, such as *Acinetobacter johnsonii*, accumulate up to 30% of dry cell weight [21]. PolyP granules are also known to be in eukaryotic cells, for example in trypanosomes [22], but are referred to as "acidocalcisomes." The polyP was observed as acidic, black electron-dense granules within calcium rich organelles [23]. These organelles are common in algae, plants [24], humans [25], and even in bacteria [26]. Prokaryotic cells generally lack endomembrane systems, so early suggestions that volutin granules were surrounded by a membrane [27] were ignored until H^+-translocating pyrophosphatase, a marker for acidocalcisomes in unicellular eukaryotes, was identified by immuno-electron microscopy in the membrane surrounding polyP granules in *Agrobacterium tumefaciens* [26]. Docampo and co-workers recently reviewed acidocalcisomes. The authors presented an explanation of the presence of acidocalcisomes in both prokaryotes and eukaryotes as being of ancestral origin; this occurred before the divergence of prokaryotes and eukaryotes; and they see a convergent evolution of the polyP granules at all basic cell types to be unlikely [28].

Microscopic localisation of polyP is important for understanding its function. In the past, Kornberg and his group reviewed and proposed various alternative cell functions for polyP [6,29], showing that not only is polyP a means of storing energy [10] but it also acts as a reservoir for phosphate [30], a chelator of metal ions [31], a buffer against alkali ions [32], a channel for DNA entry [33], a regulator of stress and survival [6] and a supportive component in gene regulation [34]. In microorganisms, polyP is directly linked to physiological processes including mobility, biofilm development, quorum sensing and virulence [35,36].

Enzymes connected to the energy metabolism of polyP are polyphosphate kinase (PPK) [10], polyphosphate: glucose-6-phosphotransferase [37], exopolyphosphatase (PPX) [38], polyphosphate: adenosine monophosphate phosphotransferase (PAP) [39], 1,3- diphosphoglycerate: polyphosphate phosphotransferase [40], tripolyphosphatase [41], polyphosphate glucokinase [42] and endopolyphosphatase [43]. PPKs are key enzymes because they are capable of shifting both energy and phosphate in both directions, storage or consumption, of phosphate-energy control. PPKs are found in bacteria, archaea [44], fungi [45], yeast [46], toxoplasma [47] and algae [48,49], yet they still remain elusive in mammalian and seed plant cells [50,51]. Although PPKs were not identified in

mammalian cell [50-52], it is accepted that production of polyP in these cells is linked to mitochondrial respiration, polyP is required for a normal function of respiratory chain, most importantly Complex IV [53,54]. There is a suggestion that a link exists between F_1F_0-ATPase regulation of polyP metabolism and mitochondrial permeability transition pore activation [50].

PPKs as energetic enzymes
PPK1
One of the important enzymes in biosynthesis and degradation of polyPs is polyphosphate: ADP phosphotransferase, referred to as polyphosphate kinase (PPK); it was first found in *E. coli* bacteria by Kornberg [10]. *Ec*PPK (EC 2.7.4.1) is a homotetramer that contains subunits with a molecular mass of 80 kDa [55]. The enzyme is bound to cell membranes [56] and catalyses polymerization of the terminal phosphate of ATP into a polyP chain [10]. This enzyme is referred to as polyphosphate kinase 1 (PPK1) [57]. It has been discovered that the enzyme accepts all nucleotide diphosphates (NDPs) and uses a polyP chain as a phosphate donor; it shows preference for purine nucleotides. The phosphorylation efficiency of NDP substrates is as follows: ADP > GDP > UDP > CDP [58]. *Ec*PPK1 possesses a V_{max} of 3700 U/mg protein (polyP$_{750}$ degradation and ADP phosphorylation) and a value of 0.073 for the ratio (polyP$_{750}$ degradation, ADP phosphorylation)/(polyP synthesis, accepting polyP$_{15}$ and ATP) [57].

$$NTP + polyP_n \leftrightarrow NDP + polyP_{n+1}$$
$$NDP : ADP > GDP > UDP > CDP$$
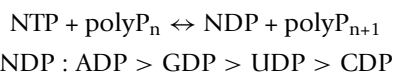
PPK2
Scientists have attempted to characterise PPK1 in other organisms, but null mutants of *Pseudomonas aeruginosa* PAO1, without detectable PPK1 activity levels, still possess as much as 20% of the wild-type polyP [59]. It has been revealed that a novel enzyme PPK2, which phosphorylates GDP to GTP by using polyP as a donor [57], is coded by the PA0141 gene [44]. It was also found that PPK2 could use GTP or ATP in the synthesis of polyP chains, differing from PPK1, which exclusively use ATP [57]. *Pa*PPK2 poses a V_{max} 500 000 U/mg protein (polyP$_{15}$ degradation and GDP phosphorylation) and a value 75 for the ratio (polyP$_{15}$ degradation, GDP phosphorylation)/(polyP synthesis, using GTP) [57].

$$NTP + polyP_n \leftrightarrow NDP + polyP_{n+1}$$
$$NDP : GDP, ADP$$
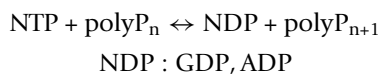
In 2008, Nocek and colleges found that many genomes encode 2 or 3 paralogs of PPK2; most of them are 1-domain PPK2s, which are about 230 residues in length. Some genomes show the presence of a longer gene with 496-544 residues, probably produced by gene duplication, and these genes produce the 2-domain PPK2. For example, the genome of *P. aeruginosa* encodes two 1-domain PPK2s (PA0141 and PA2428) and one 2-domain PPK2 (PA3455). The authors purified some 1-domain PPK2s and some 2-domain PPK2s and found that all 1-domain PPK2s exhibited polyP-dependent ADP phosphorylation activity and generated ATP, while all 2-domain PPK2s catalysed polyP-dependent phosphorylation of AMP and produced ADP. This activity, which generates ADP, is characteristic of polyP: AMP phosphotransferase (PAP) from *Acinetobacter johnsonnii* (210AA). The authors showed that the PAP protein shares a 40% sequence homology with PA3455 and contains 2 fused PPK2 domains, indicating that it is a 2-domain PPK2 [60].

PPK3
Using a BLAST search, we identified over 500 homologs of *P. aeruginosa* PPK2 (PA0141) with distributions from 1 to 6 homologs of PPK2 in one species [61]. We selected for research *Silicibacter pomeroyi*, including 3 homologs of PPK2 [61]. The genes were cloned into *E. coli* and, after yield-activity characterisation, the first PPK2 homolog (SPO0224) revealed properties similar to *E. coli* PPK1, the second PPK2 homolog (SPO1256) revealed properties similar to *P. aeruginosa* PPK2 and the third PPK2 homolog (SPO1727) showed a distinguishing selectivity for pyrimidine NDPs [61]. We named the SPO1727 polyphosphate kinase 3 (PPK3) [61]. PPK3 uses inorganic polyP as a donor to convert CDP to CTP [61]. PPK3 can phosphorylate NDP substrates as follows: CDP > UDP > GDP > ADP. The efficiency of polyP utilisation was found to vary among the different *Sp*PPKs. *Sp*PPK2 (SPO1256) and *Sp*PPK3 (SPO1727) utilised 100% of polyP while *Sp*PPK1 (SPO0224) utilised only 30%. These results led us to hypothesise that *S. pomeroyi* uses *Sp*PPK1 (SPO0224) for polyP synthesis and energy storage while *Sp*PPK2 (SPO1256) together with *Sp*PPK3 (SPO1727) is used for polyP utility [61].

$$NTP + polyP_n \leftrightarrow NDP + polyP_{n+1}$$
$$NDP : CDP > UDP > GDP > ADP$$
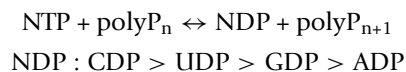
Based on this functionality, we proposed to classify polyphosphate kinases as PPK1 (poly P synthesis), PPK2 (poly P degradation with purine phosphorylation), and PPK3 (poly P degradation with pyrimidine phosphorylation). These three classes can be doubled when 2-domain PPKs are also considered (NMP-phosphorylation). This is different from protein sequence

classifications, where we recognize *E. coli* PPK1 homologs (*Ec*PPK1), *P. aeruginosa* PPK2 homologs (*Pa*PPK2) and homologs of *S. cerevisiae* vacuolar transporter chaperone's (VTC) complexes (*Sc*VTCs).

*Ec*PPK1, *Pa*PPK2 and *Sc*VTC4p molecular structures (see Figure 2)

Knowing the structures of PPKs can help us understand the functions of PPKs and also the origin and evolution of the enzymes. However, many authors have varying opinions. Some authors see similarities between *Ec*PPK1 and other polymerases such as the ribosome or RNA polymerases because everyone synthesised chains inside the tunnel [62]. They think that the *Ec*PPK1 structure may shed light on polymerase evolution as *Ec*PPK1 can be characterised as a polymerase without a template [62]. *Ec*PPK1 is also a histidine kinase because the enzyme can phosphorylate histidine residues during autophosphorylation. However, no structural similarities between *Ec*PPK1 and other histidine kinases has been found. Some structural similarities were found within the catalytic domains of phospholipase D and lipid phosphatase. The *Ec*PPK1 structure contains two asymmetric units [62] related by a pseudo two-fold symmetry that form an interlocked dimer structure; one asymmetric unit contains two monomers. Each monomer has a molecular mass of 80 kDa with 687 amino acids [55,62] and shows an L-shaped structure with four structural domains as follows: amino-terminal domain
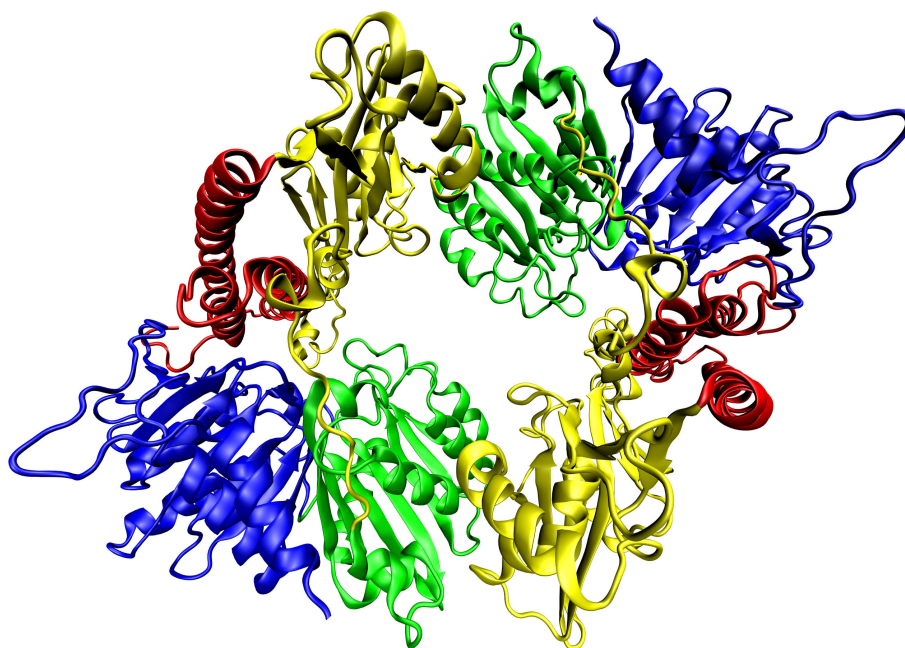
(N domain) coloured in red, the "head" domain (H domain) in yellow and two carboxyterminal domains (C1 and C2 domains) in green and blue (Figure 3) [62-65]. The N domain lies on the upper surface of the C terminal domains, consisting of 2-106 residues and forming three long antiparallel α-helices. The H domain contains 107-321 residues and forms two α-helices and a β-sheet between them; it forms the outward facing "head" of the monomer and interacts with the C1 domain. Both the C1 and C2 domains (residues 322-502 and 503-687) contain seven-stranded mixed β-sheet flanked by five α-helices [62].

The C1 domain is important for the first step of polyP synthesis, which involves the autophosphorylation of *Ec*PPK1 histidine residues. It was found that of the 16 histidine residues in *Ec*PPK1, 4 are conserved [62]. Mutagenesis of these 4 conserved His residues show that 2 (His-435, His-454 [62] or numbered as His-441, His-460 [66]) are important for autophosphorylation of enzymatic activity and polyP accumulation in the cell [55,66]. However, His-454 is totally buried within the hydrophobic core of the C1 domain, suggesting that His-435 is the only autophosphorylation site for *Ec*PPK1. One proposed model of autophosphorylation is that the γ-phosphate group of ATP attacks via His-435. His-592 functions as an acid, promoting the oxygen atom between the β- and γ-phosphate [62]. We recognised four conserved amino acids Glu-623, His-435, Asp-470, His-592 of the C1 and C2 domains *Ec*PPK1
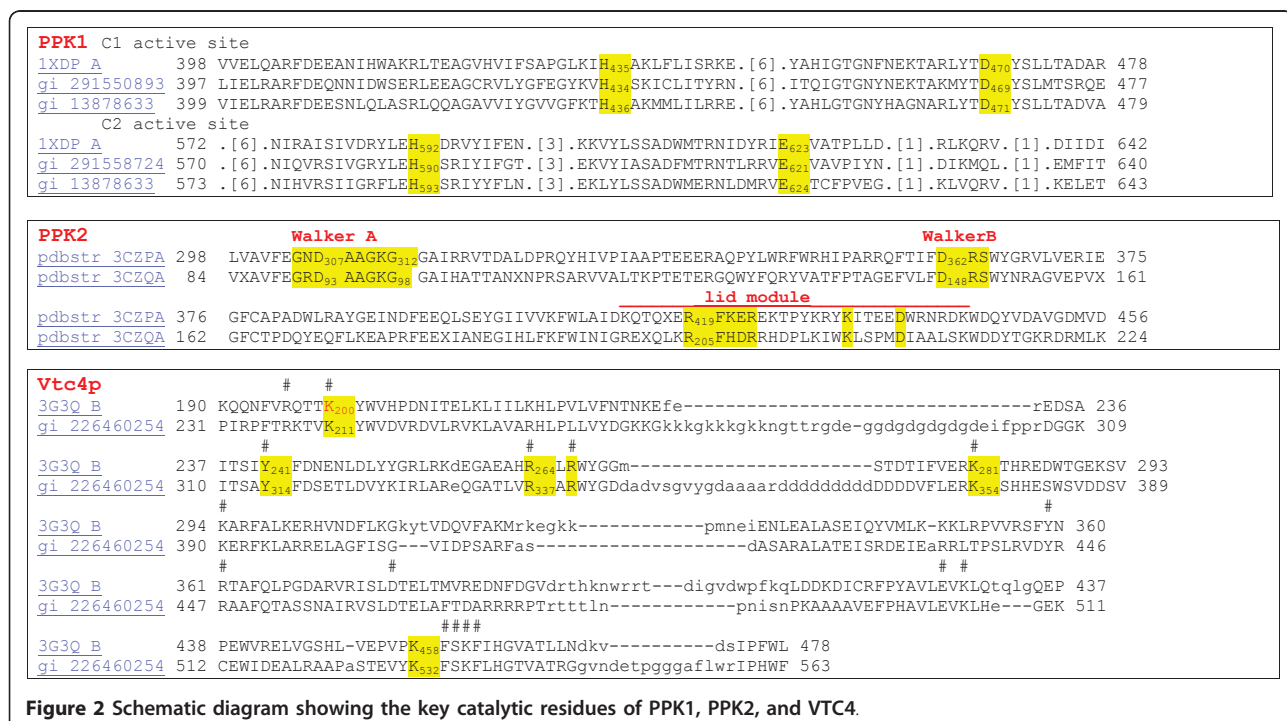
**Figure 2 Schematic diagram showing the key catalytic residues of PPK1, PPK2, and VTC4.**

Figure 3 Crystal structure of *E. coli* asymmetric unit polyphosphate kinase 1 [62]**and its implication for polyphosphate synthesis**. PPK1 contains two asymmetric units. On the picture, there is an asymmetric unit of PPK1, which contains two monomers. Each monomer of PPK1 contains amino-terminal domain (N domain) coloured in red, the "head" domain (H domain) in yellow and two carboxyterminal domains (C1 and C2 domains) in green and blue. The coordinates were downloaded from Protein Data Bank (the corresponding PDB code- 1XDO) [63] and visualized by Visual Molecular Dynamics 1.9 [64] and POV-Ray [65].

that form crucial hydrogen bonds. The amino acid Glu-623 interacts with His-435 and likely plays a role in selecting the correct rotamer of His-435 by lowering the pK_a and attacking ATP. The amino acid Asp-470 interacts with His-592 and likely facilitates in providing the correct orientation of His-592 [62]. After phosphorylation of *Ec*PPK1, the enzyme is ready to synthesise polyP chains; this process runs in a highly conserved structural tunnel, with the tunnel penetrating the centre of each *Ec*PPK1 monomer. One side of the tunnel contains a highly hydrophobic pocket that accommodates one ATP molecule, and all three phosphates are coordinated by two magnesium ions [55,62]. The other side of the tunnel contains highly conserved, positively charged residues that interact with polyP chains during elongation. It is plausible that ATP enters from one side of the tunnel and polyP chains exit from the other side [62].

*Pa*PPK2 shows structural similarities with thymidylate kinases. The conservation of key catalytic residues of thymidylate kinases in *Pa*PPK2 homologs suggests that these enzymes have a common evolutionary origin and catalytic mechanism [60]. Nocek and colleagues assembled crystal structures of PA3455 and SMc02148. They found that PA3455 is *Pa*PPK2 and has homodimeric organisation with a molecular mass of 97 kDa. Each monomer contains two PPK2 domains (residues 1-

238 and 259-495) coloured in yellow and green connected by a flexible linker (residues 238-258) coloured in red (Figure 4) [60,63-65]. SMc02148 is a *Pa*PPK2 homolog from *Sinorhizobium meliloti* and contains four PPK2 monomers in an asymmetric unit with a molecular mass of 124.5 kDa (Figure 5) [60,63-65]. PA3455 and SMc02148 monomers have similar structures, with both containing N- and C- terminal PPK2 domains. Each domain contains a 3-layer $\alpha/\beta/\alpha$ sandwich and 5 (PA3455) or 6 (SMc02148) parallel β sheets in the central location of the domain. The central β-sheet is flanked by 3 longer sheets on one side, 5 shorter sheets on the other side and 2 α-helixes at the top of the C terminal for the PA3455 domain; for the SMc02148 domain, similar structure is seen. It should be noted that the N-terminal domain was only partially modelled. The authors suggest that the active side of the enzyme is under the lid module near the 2 Walker loops (Figure 2). Walker motifs contain the conserved residues Ala-309, Gly-310, Lys-311, Gly-312, Asp-362 and Arg-423 in PA3455 and Gly-96, Lys-97, Arg-209 and Lys-218 in SMc02148. The Walker A motif binds the β- and γ-phosphates of ATP and the Asp of the Walker B motif coordinates Mg^{2+} cations [60,67].

In *Saccharomyces cerevisiae*, polyP is accumulated in both the extracellular space and the vacuoles [68]. PolyP
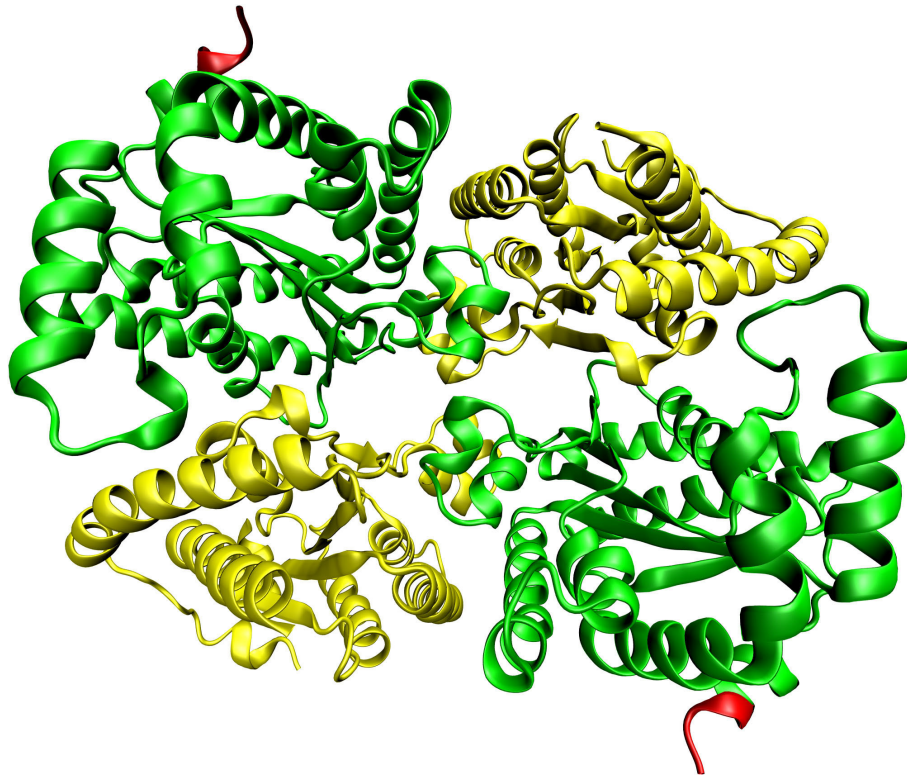
Figure 4 Crystal structures of *P. aeruginosa* polyphosphate kinase 2 [60]**and their implications for polyphosphate synthesis**. *P. aeruginosa* PPK2 contains two monomers. Each monomer contains two domains coloured in yellow and green connected by a flexible linker coloured in red. The coordinates were downloaded from Protein Data Bank (the corresponding PDB codes- 3CZP) [63] and visualized by Visual Molecular Dynamics 1.9 [64] and POV-Ray [65].
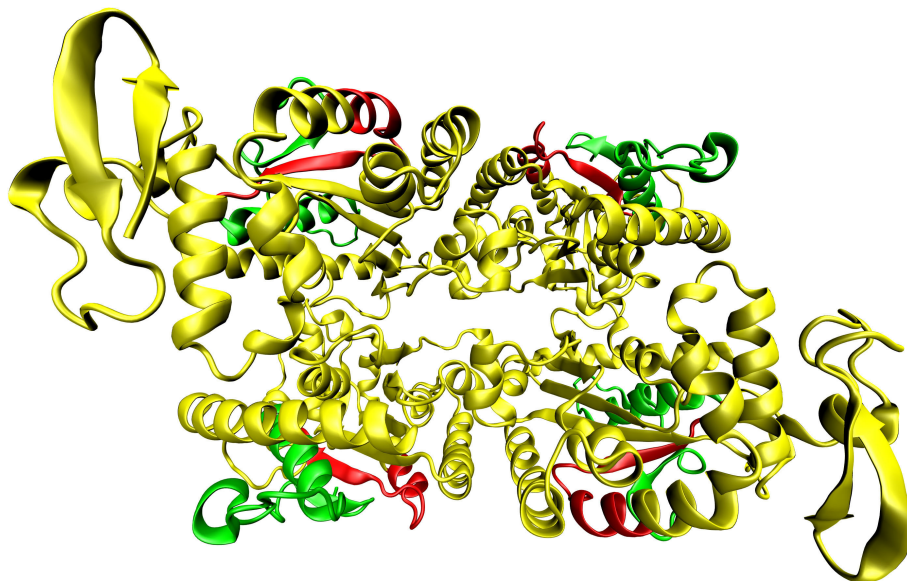
Figure 5 Crystal structures of *Sinorhizobium meliloti* polyphosphate kinase 2 [60]**and their implications for polyphosphate synthesis**. *S. meliloti* PPK2 contains four monomers. Each monomer contains two domains coloured in yellow and green connected by a flexible linker coloured in red. The coordinates were downloaded from Protein Data Bank (the corresponding PDB codes- 3CZQ) [63] and visualized by Visual Molecular Dynamics 1.9 [64] and POV-Ray [65].

synthesis is connected with the vacuolar transporter chaperone complex (VTC). VTC proteins form a membrane assembly made of hetero-oligomeric proteins [69]. It is possible to distinguish the small transmembrane protein VTC1 from the three proteins that contain transmembrane domains and a cytoplasmic segment, VTC2, VTC3 and VTC4 [70]. The most interesting protein is VTC4, which is essential for accumulation of polyP in the cell. VTC4 contains two monomers coloured in blue and red (Figure 6) [46,63-65]. The structure of VTC4 contains the tunnel that generates polyP from ATP. The entire tunnel domain contains substrate-binding and acceptor pockets. It is likely that the cleaved γ-phosphate from ATP is attached by Lys-200 and then transferred into the acceptor pocket [46].

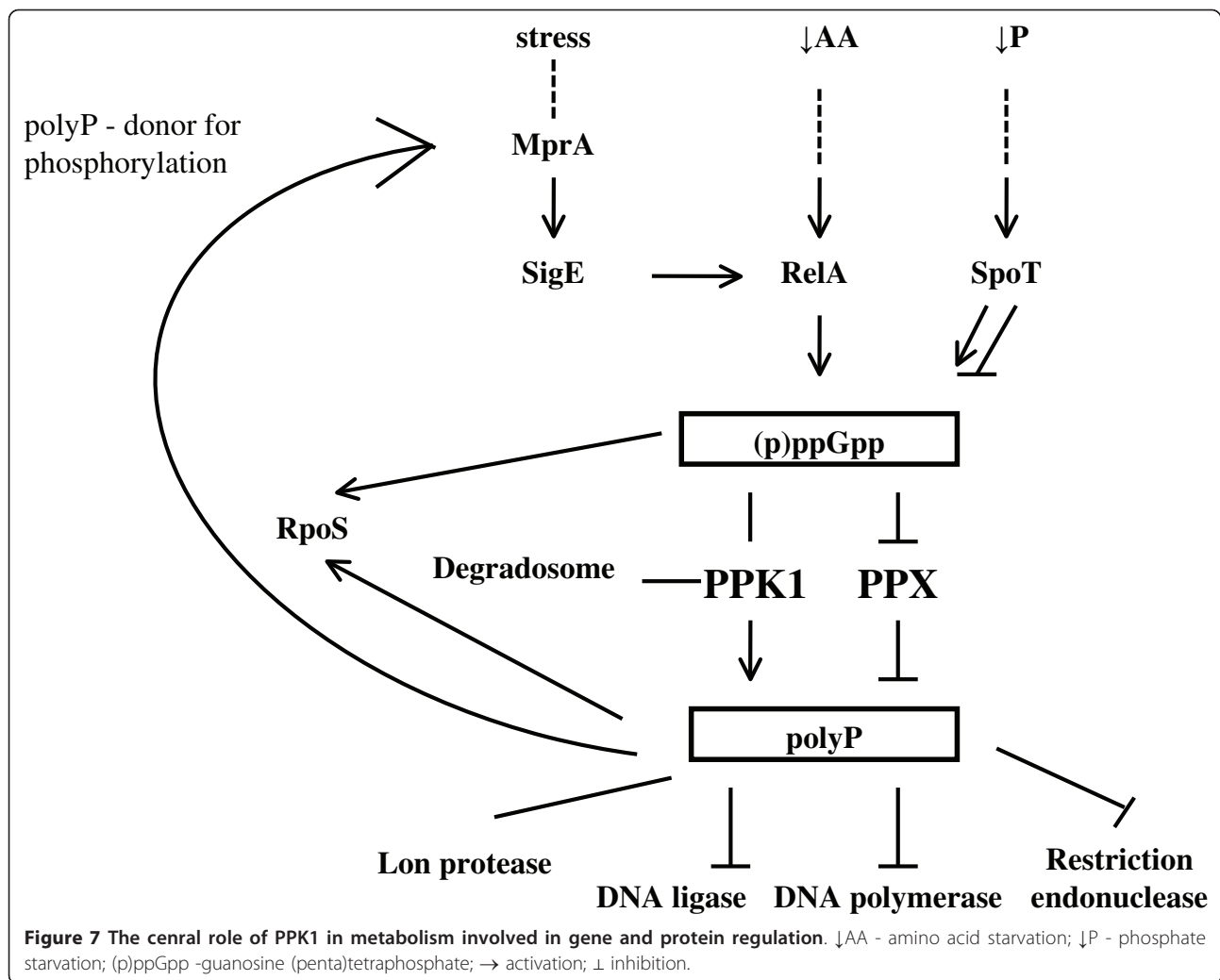## PolyP and PPK as active metabolic regulators (see Figure 7)

In *E. coli*, a model prokaryote, the Pho regulon senses low concentrations of orthophosphate in the medium. Phosphate starvation in the cell is detected by PhoR, which leads to activation of the principal phosphate regulator PhoB [71]. This regulator, in turn, activates more than 30 genes, including PhoA that encodes for alkaline phosphatase and SpoT; this in turn accumulates or degrades ppGpp [72]. Amino acid starvation in *E. coli* leads to the activation of RelA, which is responsible for a massive accumulation of guanosine (penta) tetraphosphate (p)ppGpp [73]. It has been reported that low phosphate concentrations and low concentrations of amino acids in the growth medium are required for polyP accumulation. Thus, a mutant lacking both genes, RelA and SpoT, accumulates neither (p)ppGpp nor polyP [74]. PpGpp controls bacterial transcription, translation and replication [75], so the connection

between (p)ppGpp and polyP indicates a broader role for polyP in cell regulation. For example, it has been reported that polyP plays a central role in the stress response of mycobacterium, where PPK1 is required for the MprAB-sigE-rel signalling system [76]. The presence of PPK1 leads to transcription of the two-signal transduction system MprAB, which in turn regulates the expression of SigE, a stress regulated δ^E-factor. δ^E regulates transcription of the ppGpp regulator RelA [76]. It seems that under stress conditions, polyP is a preferred donor for phosphorylation of MprA [77], a cytoplasmic response regulator. MprA then binds the promoter of the MprAB operon to initiate transcription, providing a positive feedback loop in which production of MprA brings further MprA synthesis. In this way, the MprAB operon reaches a basal level of gene expression [77,78]. If the MprAB operon is activated, phosphorylated MprA increases transcription of the gene SigE and consecutively increases transcription of Rel-ppGpp synthesis in *Mycobacterium smegmatis* and *M. tuberculosis* [76].

It has been reported that the activities of enzymes that both synthesise and degrade polyP chains fluctuate only marginally [74]. For these reasons, turnover of polyP is generated by cyclic hydrolytic breakdown by exopolyphosphatase (PPX) and synthetic accumulation by PPK [58,74]. PPX is an enzyme that degrades polyP and releases orthophosphate from the ends of polyP [38]. It has been demonstrated that pppGpp inhibits *E. coli* PPX but not *Ec*PPK, which in turn leads to a 100- to 1000-fold accumulation of polyP [58]. The coordinated regulation of *Ec*PPX and *Ec*PPK activities is not surprising. The *E. coli* genes for PPX are located downstream of Ppk and are organized in a co-linear arrangement, thus forming an operon. This means that the level of polyP degradation activity by *Ec*PPX is always dependent on
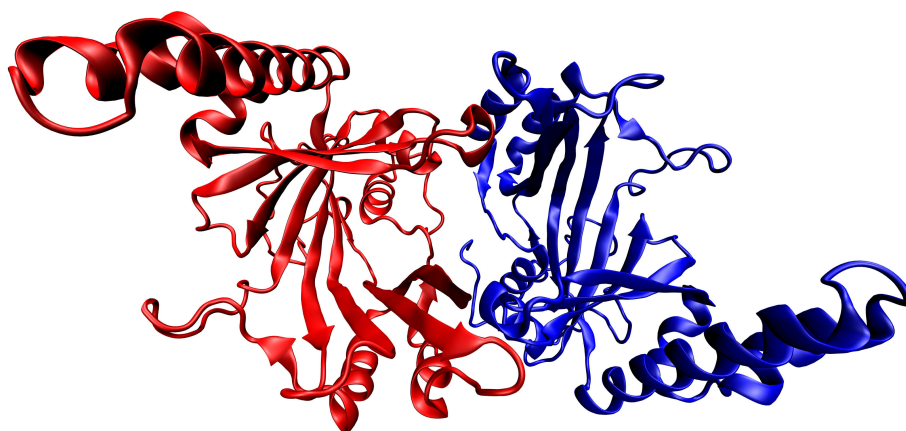
Figure 6 Crystal structure of *Saccharomyces cerevisiae* VTC4 [46]**and its implication for polyphosphate synthesis**. VTC4 contains two monomers coloured in red and blue. The coordinates were downloaded from Protein Data Bank (the corresponding PDB code- 3G3Q) [63] and visualized by Visual Molecular Dynamics 1.9 [64] and POV-Ray [65].

Figure 7 The cenral role of PPK1 in metabolism involved in gene and protein regulation. ↓AA - amino acid starvation; ↓P - phosphate starvation; (p)ppGpp -guanosine (penta)tetraphosphate; → activation; ⊥ inhibition.

the polyP synthesis level of *Ec*PPK [56]. Another example can be found in *P. aeruginosa*. The PPX homologue gene in *P. aeruginosa* is located in a direction opposite of Ppk1, and so they do not form an operon. This difference may account for the difference in polyP levels among different bacteria [79]. PolyP accumulation in *P. aeruginosa* is several-fold greater than in *E. coli* [80]. It appears that *Pa*PPX enzyme levels are regulated independently of the *Pa*PPK1 levels [79]. A microarray analysis indicated that PPK1 has a central role in gene regulation. The DNA microarrays showed changes in mRNA levels of the *P. aeruginosa ppk1* mutant; it was observed that 240 genes were up-regulated and 460 genes were down-regulated. In the case of the *P. aeruginosa ppk2* mutant, only 20 genes were up- or down-regulated [81]. Overexpression of the *E. coli* Ppk1 gene increased polyphosphate: AMP phosphotransferase (PAP) activity drastically. Investigation of this mechanism revealed that *Ec*PPK1 overproduction enhanced the activity of adenylate kinase and expressed PAP activity

[82]. PPK1 has important regulation roles in microbial cells and it is not found in higher eukaryotes. Thus, PPK1 has been suggested as a potential target for antibiotics [83].

PolyP, PPK and mRNA connections

As mentioned above, cell starvation of phosphate, nitrogen, amino acids and other nutrients induces a stress response signal that generates (p)ppGpp [73,84]. These nucleotides repress many genes, including those for ribosome synthesis, and activate 50 or more genes responsible for coping with stress and starvation [85]. Accumulation of (p)ppGpp in *E. coli* plays a major regulatory role in synthesis of the stationary-phase specific RNA polymerase sigma factor (δ^S), which is encoded by the RpoS gene [15,86] and leads to initiation of the stationary phase [73,84]. It was reported that polyP is also necessary for induction of the transcription factor RpoS [86]. δ^S is a major player in the regulation of gene expression in the stationary phase, and is activated in

response to various stresses including nutrient limitations and osmotic challenges [87]; more than 30 genes show *RpoS*-dependent expression in *E. coli* [88].

PPK1 is a component of the *E. coli* degradosome and plays a role in mRNA degradation. *Ec*PPK1 does not bind to RNA at the 3' or 5' terminal phosphate, but has to bind along the backbone; RNA binding activity involves the active centre of the enzyme. *Ec*PPK1 may promote assembly of the degradosome, or its interaction with the RNA may maintain an appropriate microenvironment by removing inhibitory polyphosphates. PolyP is a potential inhibitor of mRNA degradation by the degradosome [89]. It was reported that, in vitro, polyP inhibits other nucleic acid-modifying enzymes such as DNA ligase, restriction endonuclease and DNA polymerase [90]. PPK can bind and degrade inhibitory polyP in the presence of ADP [89] or it can participate in the cyclic hydrolytic breakdown of polyP by PPX [58,74]. As PPK is inhibited, the mRNA half-life in vivo is decreased [90]. As PPK degrades polyP, ADP is removed. ADP is a potential inhibitor of polynucleotide phosphorylase [91] in the degradosome. Regeneration of ATP by PPK is required for RhlB helicase activity [89].

PolyP, PPK and protein connections

In *E. coli*, the degradation of most cytoplasmic proteins consumes ATP [92]; ATP-dependent protease Lon is mainly involved in this process [93,94]. Kuroda and colleagues found that during stress, the Lon protease forms a complex with polyP. The polyP-Lon complex is very large because one molecule of polyP binds to four molecules of Lon. This complex degrades free ribosomal proteins [95]. The degradation of intracellular proteins can be important in cell responses to stress; this generates free amino acids that can be used as an immediately accessible source needed in the synthesis of new stress-response proteins, such as regulatory enzymes and transporters [96,97].

PolyP and DNA compete to bind Lon. The binding sites are localised in the same ATPase domain of Lon protease, and it seems that Lon has a higher affinity for polyP than for DNA [98]. Some studies show that Lon controls the level of mRNA transcription for the *E. coli* gal operon [99]. *E. coli* Lon proteases look like DNA-binding proteins but with low specificities. The drastic change in intracellular soluble polyP levels can affect the DNA-binding ability of Lon and its regulation of cellular functions [100,101]. It was shown that polyP stimulates translation in vitro [102]. McInerney and colleges showed that polyP could also interact with intact ribosomes, where the strongest points of attachment were on the protein components of the ribosome. PolyP attaches to both the 50S and 30S subunits of ribosomes [103].

Group II introns are ribozymes as well as bacterial mobile elements thought to be ancestors of both introns (genetic material that is discarded from messenger RNA transcripts) and retroelements (genetic elements and viruses that replicate via reverse transcription) in all three domains of life. *Lactococcus lactis* catalytically activates intron RNA (Ll.LtrB) and an intron-encoded reverse transcriptase (LtrA) from ribonucleoprotein particles localized in the cellular poles of bacteria. Zhao J. and co-workers used fluorescence microscopy with cell microarrays to screen a transposon-insertion library for mutants with altered LtrA localisations. They found that LtrA localisation in the mutants was affected by the accumulation of intracellular polyP. PolyP delocalized ribonucleoprotein particles away from the cellular poles. Thus, polyP serves as a potential regulator of protein localisation with wide physiological consequences [104].

Possible PPK applications in industry

As described above, Arthur Kornberg (Nobel Prize in Physiology or Medicine, 1959), together with his wife Sylvy Ruth and Simms E. S., identified PPK for the first time in *E. coli* in 1956 [10]. In the following year, Kornberg S.R. showed the reverse reaction and proposed it was a system for ATP synthesis [105]. After 20 years, the ATP-regeneration system based on polyP and *Ec*PPK1 has been suggested for use in enzyme technology applications [106]. In this system, a reaction mixture, with ADP and polyP, is percolated through a column containing immobilized *Ec*PPK1. The ATP-enriched mixture can then be used in the next reaction [106]. However, isolation of the cell extract while maintaining high *Ec*PPK1 activities proved to be difficult; because *E. coli* cells are rich in ATP-degrading enzymes, a simple separation process from ATP-hydrolysing activities was still needed [107]. Hoffman and co-workers (1988) purified *E. coli* lysate enough to stop the ATP hydrolysis activity through ammonium sulphate precipitation and DEAE cellulose fractionation. They obtained 390 mg of *Ec*PPK1 from 1 kg of fresh cell paste and immobilised the enzyme using glutaraldehyde-activated (2-aminoethyl) cellulose, which decreased the enzymatic activity to 10.6% [107]. Production and immobilisation of the enzyme was later improved by recombinant DNA technology. His-tagged *Ec*PPK1 was easily produced and immobilised on a nickel chelating resin, yet the ATP-regeneration process was unfortunately unstable [108]. It was found that overproduction of *Ec*PPK1 in *E. coli* leads to accumulation of inclusion bodies, and that the inclusion bodies are sufficiently pure and surprisingly active [109]. When these inclusion bodies were entrapped in agar/TiO_2 beads the ATP-regeneration process was stable, and the system was again suggested for use in enzyme technology applications [109]. The

basic disadvantage of the proposed system has been the low "total turnover number" (TTN), which is the total moles of product formed per mole of cofactor during the course of a complete reaction [110]. In light of this, other ATP-regeneration systems, such as acetyl phosphate and acetate kinase [111], phosphoenol pyruvate and pyruvate kinase [112] and creatine phosphate and creatine kinase [113] proved to be more attractive for enzyme technology applications. For example, Gene-Chem, Inc. uses an acetyl phosphate and acetate kinase regeneration system for the production process of CMP-NeuAc and sialyllactose [114]. Recently, our group successfully used *S. pomeroyi* PPK3 in the same process at a laboratory scale. The characteristics of *Sp*PPK3, such as high TNT, easy immobilisation and easy separation from NTP hydrolysing activities, will hopefully lead the way to a broader spectrum of enzyme technology applications [61].

Some technological applications using thermophilic enzymes require a higher temperature resistant ATP-regeneration system. PPK from *Thermus thermophilus*, which shows a 30% amino acid sequence homology to *Ec*PPK1, generated fructose 1,6-diphosphate for at least one week at 70°C [115]. Sato and colleges studied ATP-requiring D-amino acid dipeptide synthesis using PPK from *Thermosynechococcus* [116], but this enzyme was less thermostable than *Thermus thermophilus* PPK [115].
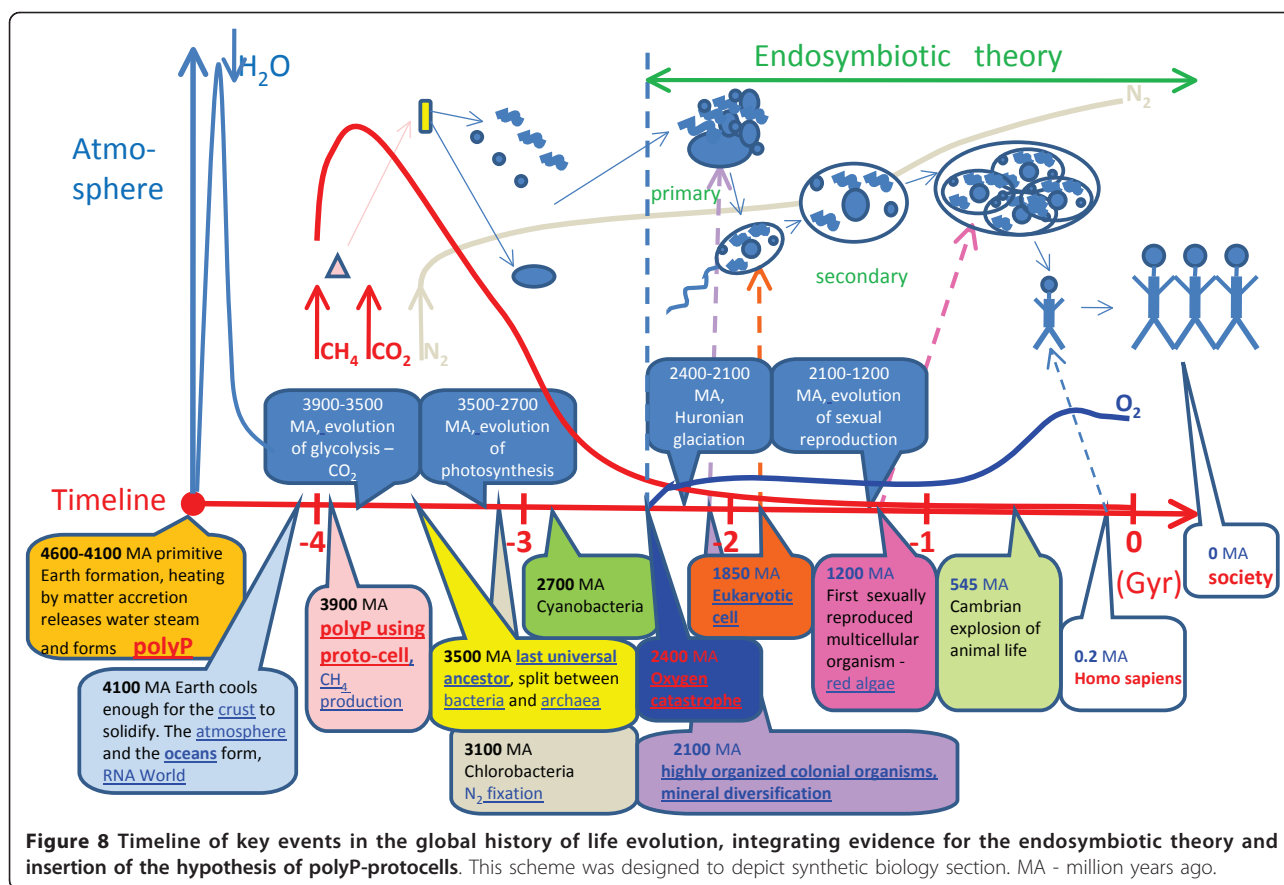
Applications in synthetic biology

"Synthetic biology" is a scientific area that includes two intentions. One area uses unnatural molecules to reproduce emergent behaviours in natural biology with the goal of creating artificial life. The other area seeks interchangeable parts from natural biology to assemble systems that function unnaturally [117]. In both cases, the intentions are focused on a better understanding of life and on the use of knowledge for a commercial benefit. For example, the design and construction of minimal cells, one main goal of synthetic biology [118], would be beneficial for the biotechnology industry. Steps towards this have already been performed; a chemically synthesised genome was successfully transplanted into *M. capricolum* bacterial cells [119]. Designing and programming synthetic life forms based on new DNA software, which includes the use of new cell materials, components and metabolic schemes, is a process coming in the near future. In terms of simplicity of the minimal genome, polyP represents an ideal energy source to power all vital functions within a synthetic cell. It could completely replace photosynthesis, respiration, glycolysis and other alternative energy sources within the cell. As mentioned above, polyphosphate is present in all natural life forms, but none use it as an essential energy source. To explain this phenomenon, this review examined

some logical references that led to a hypothesis that polyP is a molecule connected to life creation. PolyP could have been abiotically accumulated at high temperatures and under anhydrous conditions during formation of the primitive Earth, when the accretion of material heated the core and released steam to the atmosphere. The presence of acidocalcisomes in both prokaryotes and eukaryotes represents the ancestral origin before the divergence of prokaryotes and eukaryotes. Moreover, the membrane surrounding acidocalcisomes inside prokaryotic cells could have endosymbiotic origins (Figure 8). The endosymbiotic hypothesis is almost as old as Darwin's theory. Some botanists observed structural similarities between chloroplasts and *Cyanobacteria* at the end of 19th century. However, only Lynn Margulis-Sagan shifted it to theory in 1967 [120], and only recently has supportive evidence been found. The theory argues that eukaryotic cells originated from highly organized colonial organisms in connection with an "oxygen catastrophe" and "huronian glaciations" (Figure 8) 2400-2100 million years ago (MA). The prokaryotes and archaea had to react to the atmospheric poisoning by oxygen and to the age of the "snowball Earth". This supports the adaptationist model of evolution [121] where the model inevitably leads to the concept of "progress" (i.e., gradual improvement of "organs"). In evolution, successful events are conserved and integrated into developmental mechanisms, so it is no surprise that the establishment of the eukaryotic cell led to secondary endosymbiosis; this provided massive gene transfer between eukaryotes [122] and, eventually, the process led to sexual reproduction and multicellular organisms (Figure 8). The multicellular organism represents a society of highly differentiated cells with sophisticated intercommunication languages. Development of human society with languages is nothing new; the evolutionary processes remain, essentially, the same throughout the history of life. For example, even the social collective behaviour could be integrated into the evolutionary processes much earlier on the bacterial [123], viral, or gene levels. Considering this, the integration of endosymbiotic principles into the evolutionary process could be extrapolated deep into the past. We propose a model where polyP granules, or acidocalcisomes, have an ancestral endosymbiotic origin (Figure 8). If this is true, it will strongly support the sense of construction of proto-cells which use polyP as an ancient source of energy. Only two genes are needed for polyP utilisation, namely PPK and PPX. This is very interesting for design of minimal cells, a main goal of synthetic biology.

Conclusion

Inorganic polyP was probably present on Earth at the prebiotic time. At present, it is a molecule of many

Figure 8 Timeline of key events in the global history of life evolution, integrating evidence for the endosymbiotic theory and insertion of the hypothesis of polyP-protocells. This scheme was designed to depict synthetic biology section. MA - million years ago.

functions involved in energetic metabolism and gene regulation. These two processes are orchestrates by PPK enzymes that can mediate the synthesis and degradation of polyP chain.

This review prepared some references for the response why polyphosphate is present in all natural life forms. Conclusion leads to a hypothesis that polyP is a molecule connected to life creation. PolyP could have been abiotically accumulated at high temperatures and under anhydrous conditions during formation of primitive Earth; the catalytic core inside the tunnel structure of PPK could be characterized as a RNA polymerase without the template; polyP and PPKs are connected with RNA and protein regulation; and the membrane surrounding of acidocalcisomes inside the prokaryotic cell could imply endosymbiotic origin. These indications could be used in synthetic biology and microbial technologies for a minimisation of the genomic software. Besides, *Sp*PPK3 has already demonstrated to be well NTP-regeneration system (cheap substrate and high TTN - total moles of product formed per mole of cofactor) that hopefully will initiate a broader spectrum of enzyme technology applications.

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Authors' contributions
All authors read and approved the final manuscript.

Competing interests
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