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REVIEW

Inorganic polyphosphates in extremophiles and their possible functions

Alvaro Orell · Claudio A. Navarro · Matías Rivero · Juan S. Aguilar · Carlos A. Jerez

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Abstract Many extremophilic microorganisms are poly-extremophiles, being confronted with more than one stress condition. For instance, some thermoacidophilic microorganisms are in addition capable to resist very high metal concentrations. Most likely, they have developed special adaptations to thrive in their living environments. Inorganic polyphosphate (polyP) is a molecule considered to be primitive in its origin and ubiquitous in nature. It has many roles besides being a reservoir for inorganic phosphate and energy. Of special interest are those functions related to survival under stressing conditions in all kinds of cells. PolyP may therefore have a fundamental part in extremophilic microorganism's endurance. Evidence for a role of polyP in the continued existence under acidic conditions, high concentrations of toxic heavy metals and elevated salt concentrations are reviewed in the present work. Actual evidence suggests that polyP may provide mechanistic alternatives in tuning microbial fitness for the adaptation under stressful environmental situations and may be of crucial relevance amongst extremophiles. The enzymes involved in polyP metabolism show structure conservation amongst bacteria and archaea. However, the lack of a canonical polyP synthase in Crenarchaea, which greatly accumulate polyP, strongly suggests that in this phylum a different enzyme may be in charge of its synthesis.

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A. Orell · C. A. Navarro · M. Rivero · J. S. Aguilar · C. A. Jerez (✉)
Laboratory of Molecular Microbiology and Biotechnology, Department of Biology, Faculty of Sciences, Millennium Institute for Cell Dynamics and Biotechnology, University of Chile, Santiago, Chile
e-mail: cjerez@uchile.cl

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Introduction

Currently described as ubiquitous and versatile molecules, inorganic polyphosphates (polyP) are linear polymers consisting of dozens to hundreds of orthophosphate residues (Pi) linked by high-energy phosphoanhydride bonds. The possible prebiotic origin (Yamagata et al. 1991) together with a set of physicochemical features have placed polyP as a "key" molecule within early course of life evolution (Kulaev and Kulakovskaya 2000). At first, polyPs were classically considered as energy and phosphate reservoirs. However, their regulation and function remained unknown for many years due to lack of specific analytical methods. First descriptions of polyP realized the presence of metachromatic granules in microorganisms. These particles, which stained pink with basic dyes, were called "volutine" (Meyer 1904). Afterwards, by means of electron microscopy analysis, polyP were described as electron-dense granules that quickly disappeared under the electron beam, thus differentiating from chromatin (Wiame 1947). Using this approach, polyP granules have been found in all living beings: bacteria, fungi, protists, plants and animals cells (Wood and Clark 1988; Kornberg et al. 1999; Seufferheld et al. 2008; Achbergerová and Nahálka 2011) and in archaea (Scherer and Bochem 1983; Rudnick et al. 1990; Andreeva et al. 2000; Remonsellez et al. 2006).

Although in bacteria polyP are mainly cytoplasmic, they have been also found in association with the plasma membrane forming a complex with poly-beta-hydroxybutyrate (PHB) and calcium (Reusch and Sadoff 1988).

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PolyP metabolism is mainly driven in bacteria by two enzymes. Polyphosphate kinase 1 (PPK1) that catalyzes the reversible conversion of terminal phosphate of ATP into polyP: nATP ↔ polyPn + nADP. The conversion of ADP back to ATP is obtained in excess of ADP. The second enzyme is the exopolyphosphatase (PPX) that processively hydrolyzes terminal residues of polyP to release Pi (Kornberg et al. 1999). These enzymes have been purified from *E. coli* and their genes have been identified in several bacteria, including the acidophilic *Acidithiobacillus ferrooxidans* (Vera et al. 2003). These genes show a relatively high degree of sequence conservation (Tzeng and Kornberg 1998; Cardona et al. 2002).

There is strong evidence supporting that polyP plays a role in the physiological adjustments of bacteria to environmental changes and stress conditions. For example, bacterial mutant strains that lack polyP (*ppk*-null) survive poorly during growth in the stationary phase and are less resistant to heat, oxidants, osmotic challenge, antibiotics and UV radiation (Crooke et al. 1994; Rao and Kornberg 1996; Tsutsumi et al. 2000; Kim et al. 2002). PolyP accumulation in response to nutrient deprivation has also been reported in many neutrophilic bacteria, and several studies have demonstrated that PPK is essential for cell motility, biofilm development, quorum sensing, and virulence (Rashid and Kornberg 2000; Rashid et al. 2000a, b). Moreover, evidence has been accumulated indicating that polyP displays a multiplicity of biological functions depending on the organism or subcellular localization (Kornberg et al. 1999; Kulaev and Kulakovskaya 2000). These features include: substituent of ATP in kinase reactions, serving as a chelator of metals, as a buffer against alkali and playing roles during physiological adjustment to growth (Rao and Kornberg 1996; Kornberg et al. 1999; Rao et al. 2009; Varela et al. 2010).

Thus, the dynamics of accumulation and utilization of polyP reported in several biological systems clearly shows that the roles of these molecules are beyond being a source of Pi and energy reserves. In addition, the association of polyP and adaptation to environmental stress situations, by tuning microbial fitness, may be of great relevance within extremophilic microorganisms. Hereby we focus on the role that polyP may play during environmental adaptations in some extremophilic bacteria and archaea. In particular, the potential function of polyP in heavy metal ions detoxification systems in acidophilic metallotolerant bacteria and archaea is revisited.

Presence of polyP in extremophilic microorganisms

Most studies relating stress responses to polyP have been conducted using model bacteria which are not extremophiles (Chávez et al. 2004; Seufferheld et al. 2008; Rao et al. 2009). However, several extremophilic microorganisms have been reported to contain polyP granules. The occurrence of polyP in archaea was first reported in the methanogens *Methanosarcinae* and related species during growth on both methanol in an optimized medium and acetate and H_2/CO_2. PolyP-like granules were described to also contain the elements Ca, and Fe, and sometimes Mg, S, and Cl (Scherer and Bochem 1983).

Several biomining microorganisms have also been shown to accumulate electron-dense granules composed of polyP as seen in the bacterium *A. ferrooxidans* (Alvarez and Jerez 2004) and the archaeon *Sulfolobus metallicus* (Remonsellez et al. 2006). Figure 1 shows some examples of the typical sponge-like polyP granules seen by electron microscopy in selected extremophilic microorganisms. While polyP granules are clearly visible in *S. metallicus* and *Metallosphaera sedula*, *S. solfataricus* shows very small granules if any (Fig. 1). Moreover, polyP granules

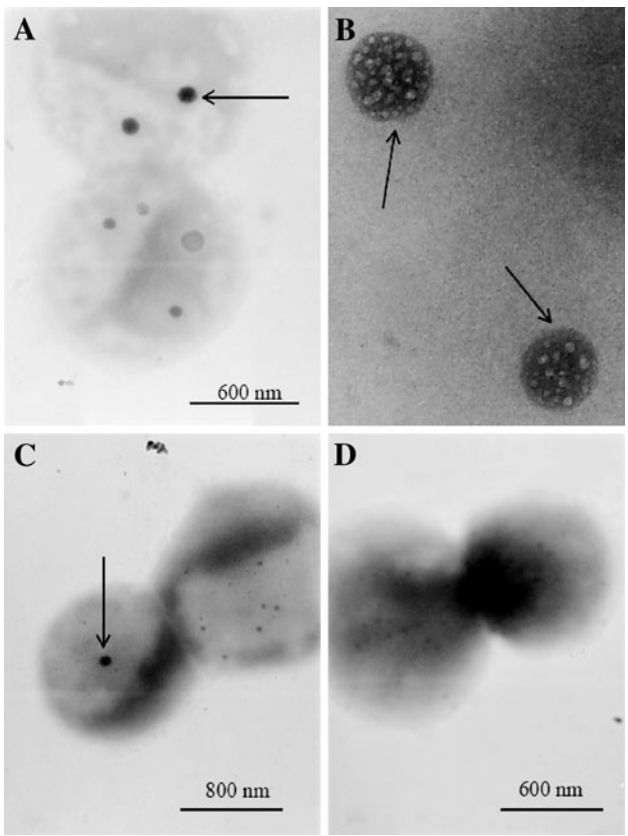

Fig. 1 Presence of polyP granules in some extremophilic microorganisms. Transmission electron microscopy of unstained cells of different thermoacidophilic archaeons. PolyP granules appear as dense bodies to the passing of electrons and are indicated by *arrows*. **a** *S. metallicus*. **b** An enlargement of a couple of polyP granules to show their characteristic sponge-like appearance. **c** *M. sedula*. **d** *S. solfataricus*

have been also described in other biomining microorganisms such as *A. thiooxidans* and *A. caldus* (Orell et al. 2010).

The presence of one or more polyP granules correlates very well with the biochemically determined levels of polyP in acidophilic bacteria and archaeons as seen in Table 1. The chemical nature of the electron-dense bodies corresponded to polyP as determined by the electron energy loss spectroscopy (EELS) procedure and it was quantified by using specific radioactive and non-radioactive enzymatic assays developed by Kornberg's group (Kornberg et al. 1999) in *S. acidocaldarius, S. metallicus* and *S. solfataricus* (Remonsellez et al. 2006) and by energy dispersive X-ray microanalysis (EDAX) in *A. ferrooxidans* (Alvarez and Jerez 2004). All four acidophilic microorganisms synthesized polyP during growth, but only *S. metallicus* and *A. ferrooxidans* greatly accumulated polyP granules. The differences in the capacity to accumulate polyP between these microorganisms may reflect adaptive responses to their natural environment. In this regard, some interesting correlations between copper resistance and polyP levels can be distinguished in these microorganisms. Accordingly, *A. ferrooxidans* synthesizes 400 nmol of polyP/mg of protein and tolerates up to 800 mM copper sulfate; *S. metallicus* that accumulates 180 nmol of polyP/mg of protein was able to grow and tolerate up to 200 mM copper sulfate. On the other hand, *S. solfataricus* that synthesizes 20 nmol of polyP/mg of protein could not grow in or resist more than 1–5 mM copper sulfate (Table 1). A working model explaining this potential relationship between copper resistance and the polyP levels found intracellularly is discussed below.

Other metal resistant microorganisms have also been reported to contain abundant polyP levels. An *Arthrobacter* spp. resistant to uranyl ion $(UO_2)^{+2}$ isolated from a uranium-contaminated site accumulated uranium intracellularly as precipitates closely associated with polyP granules (Suzuki and Banfield 2004). The authors suggested on this basis that the sequestration of uranium into polyP may correspond to a detoxification mechanism.

In addition, *Thiomonas* sp. 3As, a moderate acidophilic bacterium isolated from an extreme environment such as arsenic-rich acid mine drainage has also been reported to possess polyP granules. Presumably, this microorganism may obtain cytoplasmic buffering by using its polyP granules (Arsène-Ploetze et al. 2010).

Table 1 PolyP levels and copper resistance in acidophilic Bacteria and Archaea

Microorganism	PolyP levels (nmol of Pi/mg of protein)	Copper MIC (mM)
A. ferrooxidans	400[a]	800[c]
S. metallicus	180[b]	200[b]
S. solfataricus	20[b]	1[b]
S. acidocaldarius	10[b]	<10[d]

PolyP levels were enzymatically quantified as described by Ault-Riché and Kornberg (1999)

[a] Alvarez and Jerez (2004)

[b] Remonsellez et al. (2006)

[c] Harvey and Crundwell (1996)

[d] Miller et al. (1992)

Enzymes involved in polyP metabolism in extremophiles

A. ferrooxidans contains in its genome a putative Pho regulon including the genes *phoB, phoR, pstS, pstC, pstA, pstB, phoU, ppx* and *ppk* (Vera et al. 2003). Some differences were seen in the organization of the genes from *A. ferrooxidans* when compared with the Pho operons from other bacteria. This was especially evident in the organization of the genes involved in polyP metabolism (*ppk* and *ppx*). *E. coli* has both *ppk* and *ppx* genes in the same operon (Kornberg et al. 1999), while *A. ferrooxidans* does not (Vera et al. 2003). Therefore, if the *E. coli* genes are co-regulated at the transcriptional level, it is difficult to envisage an accumulation of polyP granules since the two enzymes have opposite activities (synthesis versus degradation of polyP). In fact, *E. coli* only transiently increases polyP synthesis under adverse conditions, and no polyP granules have been reported in wild-type strains of this bacterium. One could speculate that microorganisms having the *ppk* and *ppx* genes organized in separate operons might regulate synthesis and degradation of polyP separately, allowing them to accumulate these polymers in response to stress conditions. In this regard, an analysis of the genomic contexts of *ppk* and *ppx* genes of several other genomic sequences available was carried out using the web tool Absynte (Archaeal and Bacterial Synteny Explorer) (http://archaea.u-psud.fr/absynte/) (Despalins et al. 2011) (not shown). Thus, using the amino acid sequence of *A. ferrooxidans* ATCC 23270 PPX as query it was found that the best scores indicated that the *ppx* genes were part of putative Pho regulons and *ppk* genes were located in separate and unrelated genomic regions. These best hits were for the following acidophilic bacteria (in decreasing score values): *A. ferrooxidans* ATCC 23270 (query), *A. ferrooxidans* ATCC 53993, *A. ferrivorans* SS3 and *A. caldus* SM1. It is known that the two former and the last microorganism are able to accumulate high levels of polyP. Although these findings are limited to a reduced number of extremophiles, they support the idea that at least in these four acidophilic bacteria *ppk* and *ppx* genes are present in different loci.

While production of polyP has been proven to occur in several archaeal species (Scherer and Bochem 1983;

Rudnick et al. 1990; Andreeva et al. 2000; Remonsellez et al. 2006; Toso et al. 2011), the enzymology of polyP metabolism or the genes involved in this domain has been partially elucidated, as long as a polyP synthase activity is still to be reported and characterized. In this regard, a previous attempt to characterize the genetic components of the metabolism of polyP in the crenarchaeon *S. acidocaldarius* was done by following the published purification procedure for a glycogen-bound protein with PPK as well as glycosyl transferase (GT) activities from *S. acidocaldarius* (Skorko et al. 1989). However, the reported PPK activity for the 57-kDa protein could not be reproduced. Furthermore, no PPK activity was found associated to any of the proteins bound to the glycogen–protein complex. In addition, the previously reported PPK turned out to be highly similar to glycogen synthases from Archaea and Bacteria and also showed GT activity (Cardona et al. 2001). On the other hand, an exopolyphosphatase (PPX) gene (*ppx*) from the thermoacidophilic *S. solfataricus* was described being the first report of an enzyme to be involved in polyP metabolism in members of the Archaea (Cardona et al. 2002). The purified recombinant *S. solfataricus* PPX was highly active, degrading long-chain polyP (700–800 residues) in vitro at 50–60 °C. Bioinformatic analysis revealed that the deduced amino acid sequence of *S. solfataricus* PPX showed the highest similarity (25–45 %) to sequences of members of the bacterial-like PPXs (Cardona et al. 2002). In contrast, putative PPXs present in other archaeal genomes showed the highest similarity to yeast PPXs (Cardona et al. 2002). The crystal structure of PPX from *E. coli* at 2.2 Å resolution has been reported (Rangarajan et al. 2006). The protein forms a dimer and each dimer is made of four domains (I, II, III and IV). The structure of the protein suggests a binding mode for long polyP chains. This putative binding site of PPX is located at the interface between domain I from one monomer and domain II from the second opposite monomer (see Fig. 2). In the crystal structure of the enzyme these two domains are close together and represent the "closed" state. A very interesting feature of the dimer is a deep S-shaped canyon extending along the dimer interface and lined with positively charged residues that the authors postulate that is a likely site for polyP binding (Rangarajan et al. 2006). A structural homology model of PPX from *S. solfataricus* was done (Mobarec and Jerez, unpublished results) based on the crystal structure of the PPX from *E. coli* (Rangarajan et al. 2006). Figure 2 shows the "closed" dimer of *S. solfataricus* PPX (SsPPX) built by superposition over the closed dimer structure of *E. coli* PPX (EcPPX). Although domain IV is absent from SsPPX, the putative domains II and III that would form the suggested polyP-binding site containing basic amino acid residues appear in general structurally conserved in both structures. The lacking

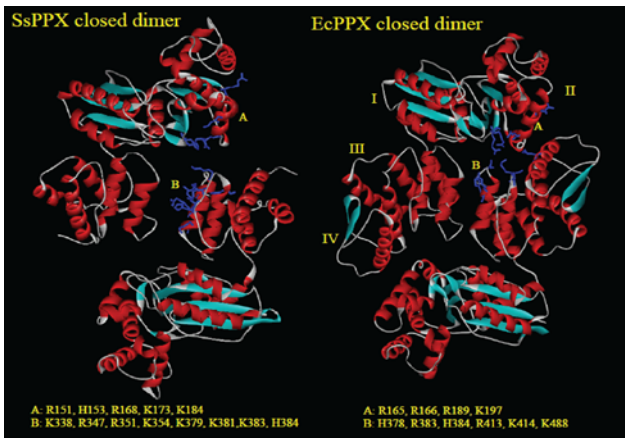

Fig. 2 Structural comparison between PPX dimers from *E. coli* and *S. solfataricus*. The homology model of *S. solfataricus* PPX (SsPPX) was done based on the crystal structure of *E. coli* PPX (EcPPX) (Rangarajan et al. 2006) using the program MODELLER (Sali and Blundell 1993). The models were selected and verified as before (Orell et al. 2010). The four domains described for *E. coli* PPX monomer (I, II, III and IV) are indicated. When both *E. coli* monomers associate into a dimer, a deep S-shaped canyon extending along the dimer interface and lined with positively charged residues has been proposed (Rangarajan et al. 2006). A similar equivalent positive canyon feature can be distinguished in the SsPPX dimer. The basic amino acid residues from chains A and B that would be within the putative positive canyons are also indicated in each protein

domain IV in SsPPX would not be essential for the enzymatic activity, since it has been previously shown that SsPPX hydrolyzes polyP in vitro (Cardona et al. 2002).

The *E. coli* PPK is a dimer containing two identical monomers. Each monomer shows an L-shaped structure with four structural domains (Zhu et al. 2005). These domains are the amino-terminal domain (N domain), the head domain (H domain) and two closely related carboxy-terminal domains (C1 and C2 domains). The crystal structure of the *E. coli* PPK monomer shows that the catalytic reactions take place in a highly conserved tunnel-shaped structure formed at the center of each monomer. Dimerization of monomers contributes to the stabilization of the tunnel-shaped structure (Zhu et al. 2005). This tunnel is formed by the intersection of domains N, C1 and C2 and a pair of helices from domain H. One side of the tunnel is able to accommodate an ATP molecule, while the other end has a series of positively charged residues, which are highly conserved. It is thought that these residues interact with the polyP which is being synthesized. The upper panels in Fig. 3 show a model for A. *ferrooxidans* PPK built by superposition over the monomeric structure of *E. coli* PPK. Both monomers contain the four equivalent domains or part of them and they are in general structurally similar so the central tunnel or suggested polyP-binding site containing basic amino acid residues appears structurally conserved in both structures (lower panels in

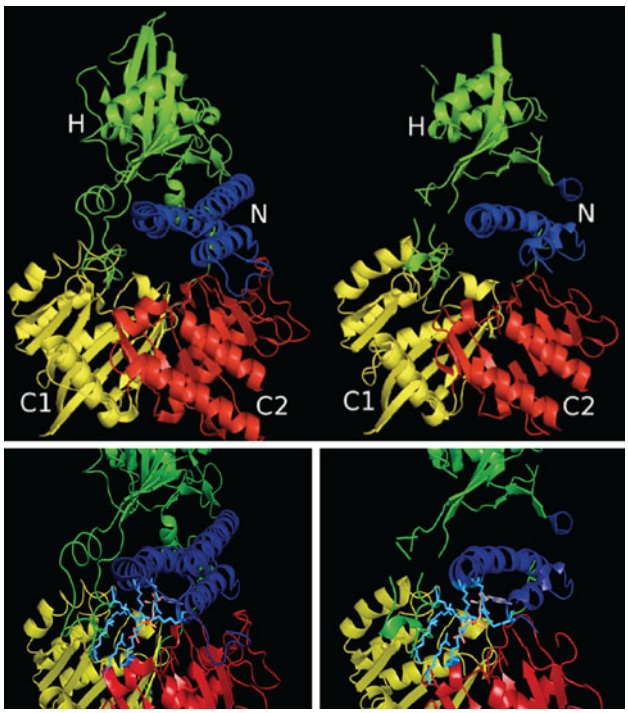

Fig. 3 Structural comparison between PPK from *E. coli* and *A. ferrooxidans*. The homology model of a monomer of *A. ferrooxidans* 23270 PPK (Afe_1876) (*upper right panel*) was done based on the crystal structure of the subunit of *E. coli* K-12 PPK (PDB: 1XDP) (*upper left panel*) (Zhu et al. 2005) using the program SWISS-MODEL workspace (Bardoli et al. 2009) and PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC. The N-terminal domains in each model are colored in *blue*, the head domains are in *green*, C-terminal domains C1 in *yellow* and C2 domains in *red*. The region at the center of each model contains the tunnel structure formed by the intersection of domains N, C1 and C2. In the *lower panels* this region is amplified to indicate the conserved basic amino acid residues (in *light blue*) and the position of an ATP molecule (in *orange*). *Left lower panel*, *E. coli* tunnel that contains K10, R53, R60, R375, R405, K433, R616 and R621. *Right lower panel*, *A. ferrooxidans* putative tunnel containing R21, R68, R75, R389, R419, K447, R627 and R632 (color figure online)

Fig. 3). Furthermore, all the residues interacting with ATP phosphate groups and those involved in side-chain contacts with the adenine ring of ATP have been reported as being highly conserved in the PPKs from *E. coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Bacillus anthracis* and *Helicobacter pylori* (Zhu et al. 2005). An alignment of the *E. coli* PPK sequence with those from the acidophilic bacteria *A. ferrooxidans*, *A. caldus*, *Thermus thermophilus* and the archaeons *Methanospirillum hungatei* and *Methanosarcina mazei* indicate the same high degree of conservation for all the amino acid residues binding ATP and those which are phosphorylated in PPK (Fig. 4). The former two bacteria and the last two archaeons have been experimentally shown to accumulate polyP granules (Scherer and Bochem 1983; Orell et al. 2010; Toso et al. 2011).

The presence of putative *ppk* and *ppx* genes was searched in the genomic sequences of some selected extremophiles. Of the archaeal genomes found at the UCSC Archaeal Genome Browser (Chan et al. 2012), only those that are extremophiles and possess only one or both of the *ppk* and *ppx* genes were selected. As seen in Table 2, all members of the Crenarchaeota possess *ppx* genes but lack a *ppk*-like gene. On the other hand, in Euryarchaeota, 9 members possessed only the *ppk* gene and one had only *ppx* (Table 2). A few extremophilic bacteria were also included for comparison. Most of them possess both *ppk* and *ppx* genes. However, the genome of the hyperthermophilic bacterium (85–95 °C) *Aquifex aeolicus* contains only a gene coding for a single protein of the PPX/GPPA family and which has been annotated as a PPX (Table 2). Still, this extremophile lacks a PPK-coding gene which suggests that the primary function of the PPX/GPPA protein is to provide *A. aeolicus* with GPPA activity (regulation of the conversion of pppGpp to the biologically effective second messenger ppGpp) (Kristensen et al. 2004). Following this idea, one could assume that all microorganisms having only a *ppx* gene would not synthesize polyP. No reports detecting polyP in *A. aeolicus* are available. However, the crenarchaeons *S. solfataricus* and *M. sedula* each containing only a *ppx*-like gene (Table 2) are able to synthesize polyP (Remonsellez et al. 2006; Orell et al. 2010). Most likely, an unknown PPK-like enzyme should exist in these microorganisms.

As already mentioned, polyP metabolism has been studied mainly in bacteria while it remains largely uncharacterized in eukaryotes. Thus, it is not well known if one or more enzymes are responsible for polyP synthesis in Eukarya. A dolichol pyrophosphate:polyphosphate phosphotransferase activity has been reported in yeast. This enzyme catalyzes the transfer of Pi from dolichol phosphate to short-length polyP chains and it is associated with cell wall glycoproteins synthesis (Kulaev et al. 1999). Despite the promising role of a dolichol pyrophosphate:polyphosphate phosphotransferase in polyP synthesis, this enzymatic activity has not been characterized to be functional in archaea. Furthermore, null mutants of four homologous genes *vtc1p*, *vtc2p*, *vtc3p* and *vtc4p* encoding for the membrane–integral vacuolar transporter chaperone (VTC) complex in *S. cerevisiae* were found to be deficient in polyP accumulation (Ogawa et al. 2000). Recently, it was shown that in *S. cerevisiae* protein Vtc4p is in charge of polyP synthesis and forms part of the VTC complex. A 2.6 Å crystal structure of the catalytic domain grown in the presence of ATP revealed also polyP winding through a basic tunnel-shaped pocket (Hothorn et al. 2009). Although the eukaryotic polyphosphate polymerase also converts ATP into polyP, there is no further significant sequence or structural similarities when compared with bacterial PPKs.

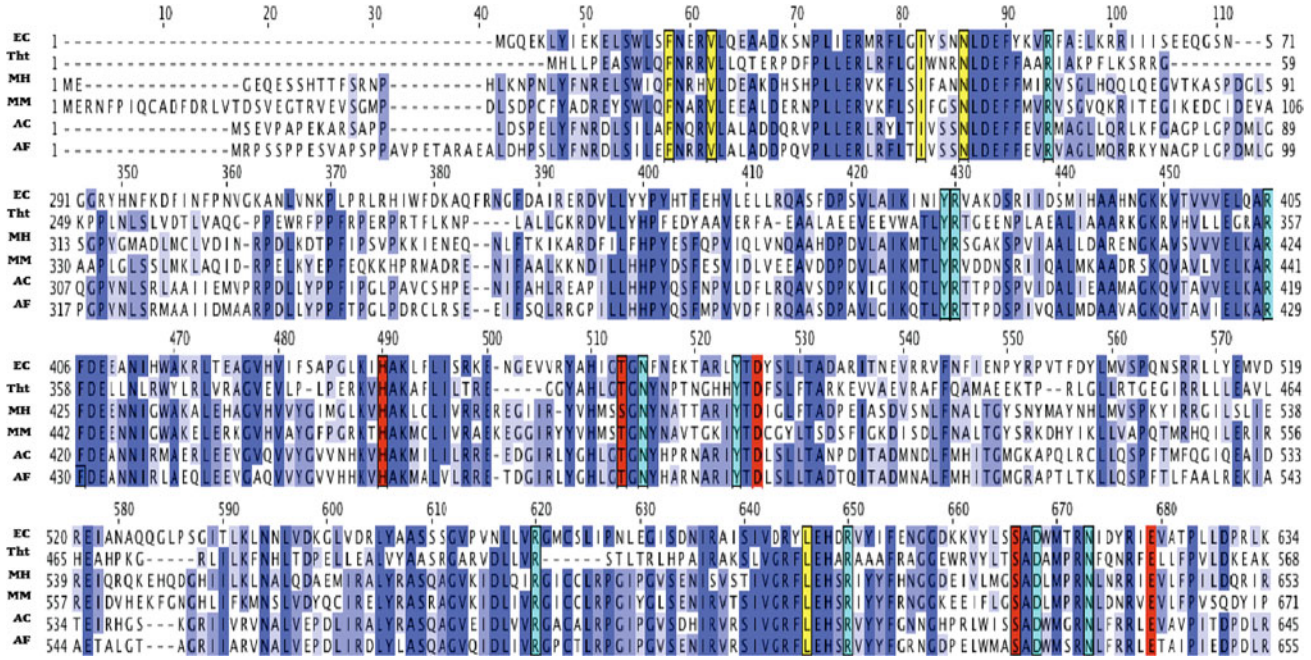

Fig. 4 Sequence alignment of polyphosphate kinases from some bacteria and archaea. Only partial sequences are aligned. Identical residues are shaded in *blue* and those partially conserved in *light blue*. Residues involved in side-chain contacts with the adenine ring of ATP are shaded in *yellow* and those involved in side-chain contacts with phosphate groups of the nucleotide are shaded in *cyan*. Residues phosphorylated or involved in autophosphorylation are shaded in *red*. EC, *Escherichia coli*; Tht, *Thermus thermophiles*; MH, *Methanospirillum hungatei*; MM, *Methanosarcina mazei*; AC, *Acidithiobacillus caldus*; AF, *Acidithiobacillus ferrooxidans* ATCC 23270 (color figure online)

It has not been possible to detect the presence of *Vtc4p* genes in any archaeal genomes by bioinformatic analyses (BLASTP and PSI-BLAST) (Navarro, Cardona and Jerez, unpublished results). Since the VTC complex appears not to be conserved in animals or plants (Hothorn et al. 2009), another class of enzymes remains to be discovered that synthesizes polyP in these organisms. In addition, a polyP biosynthetic pathway(s) remains to be elucidated in Crenarchaea and it will be of great relevance not only to reveal the biological meaning of polyP in this domain of life but also to have insights in the evolutionary changes of the enzymes involved in the synthesis and degradation of these polymers.

Potential functions of polyP in extremophiles

It is broadly described that polyP has a role in the physiological adjustments of *E. coli* and other bacteria to environmental changes and during the stationary phase of growth (Kornberg et al. 1999; Kulaev and Kulakovskaya 2000). For example, in response to nutrient limitation and during stationary phase, bacteria accumulate polyP. Furthermore, some studies in archaeal members support the presumption that polyP accumulation dynamics is in agreement with a better cell fitness to face stress situations.

Hereof, it has been described that the accumulation of polyP in *Methanosarcina frisia* was substrate dependent (cells grown on methanol accumulated more polyP than autotrophically grown cells) and far more than necessary for the maintenance of essential metabolic pathways (Rudnick et al. 1990). These results lead to the proposed hypothesis that besides their function as a Pi and energy reservoirs, and given that the synthesis of polyP is an energy-consuming process, it is not realistic to assume that they only have an osmotic function within the cytoplasm, but also an energy-delivering role that has not been directly demonstrated so far. In this regard, an archaeal polyP/ATP-dependent NAD kinase, from the hyperthermophilic *Pyrococcus horikoshii,* has been recently characterized. This enzyme utilized $polyP_{27}$ as phosphoryl donor (average length of the chain is 27) as the most active inorganic polyphosphate for NAD phosphorylation (Sakuraba et al. 2005). As a result, this might constitute an example of an energy coupled event of polyP and general cellular processes. Also, an interesting possible physical and functional relationship between carboxysomes and polyP granules has been suggested for several autotrophic bacteria (Iancu et al. 2010).

Moreover, it was found that polyP levels increase in the stationary phase of growth of *S. acidocaldarius*, *S. metallicus* and *S. solfataricus* (Remonsellez et al. 2006). Additionally, accumulation of polyP in *S. acidocaldarius* and *S. solfataricus* was observed in response to certain nutritional deficiencies as amino acid starvation (Cardona, Orell

Table 2 Presence of putative *ppk* and *ppx* genes in selected extremophilic microorganisms

	ppk gene	*ppx* gene	Growth conditions
Crenarchaea			
Acidianus hospitalis	–	Ahos_0454	Hyperthermophilic, acidophilic
Metallosphaera cuprina	–	Mcup_1197	Thermophilic, acidophilic
Metallosphaera sedula	–	Msed_0981	Thermophilic, acidophilic
Sulfolobus islandicus M.14.25	–	M1425_0368	Hyperthermophilic, acidophilic
Sulfolobus islandicus Y.N.15.51	–	YN1551_2685	Acidophilic
Sulfolobus solfataricus 98 2	–	Ssol_2163	Hyperthermophilic, acidophilic
Sulfolobus solfataricus P2	–	SSO1193	Thermophilic, acidophilic
Sulfolobus tokodaii str.	–	ST1544	Hyperthermophilic, acidophilic
Sulfolobus acidocaldarius DSM639	–	Saci_2018	Hyperthermophilic, acidophilic
Euryarchaea			
Haloarcula hispanica	HAH_5251	–	Extremely halophilic
Haloarcula marismortui	pNG7302	–	Halophilic
Haloferax volcanii	HVO_1650/HVO_0074/HVO_2598	–	Halophilic
Halomicrobium mukohataei	Hmuk_1795	–	Halophilic
Halopiger xanaduensis	Halxa_1483	–	Halophilic
Haloquadratum walsbyi	HQ1743A	–	Halophilic
Haloterrigena turkmenica	Htur_3719	–	Halophilic
Methanosarcina mazei	MM_1375	–	Adaptable to 800 mM NaCl
Methanothermococcus okinawensis	–	Metok_1097	Thermophilic
Natrialba magadii	Nmag_2061	–	Halophilic
Bacteria			
Aquifex aeolicus	–	aq_891	Hyperthermophilic
Acidithiobacillus caldus	Atc_2146	Atc_0839	Acidophilic
Acidithiobacillus ferrooxidans ATCC 23270	AFE_1876	AFE_1441	Acidophilic
Acidithiobacillus ferrooxidans ATCC 53993	Lferr_1551	Lferr_1158	Acidophilic
Deinococcus radiodurans	DR_1939	DR_A0185	Radiation resistant
Thermus thermophilus HB27	TTC0637	TTC0636	Hyperthermophilic
Thiomonas sp. 3As	Locus tag THI_2135	Locus tag THI_2134	Arsenic resistant acidophilic
Halorhodospira halophila	Hhal_0214	Hhal_0215	Extremely halophilic

A bioinformatic search was performed to check for the existence of putative *ppk* and *ppx* genes in the genomes of the microorganisms using the available genome databases UCSC Archaeal Genome Browser and GenBank. For reasons of brevity, citations for the description of the species are found in the List of Prokaryotic Names with Standing in Nomenclature, http://bacterio.cict.fr

and Jerez, unpublished observations). PPX activity of crude extracts from *S. solfataricus* was higher during the exponential phase of growth than during the stationary phase in accordance with the observed levels of polyP (Cardona et al., 2002). In conclusion, the increase of polyP levels in response to nutrient limitations suggests that polyP metabolism might be regulated by genetic components and may reflect adaptive responses to their natural environment.

On the other hand, *Methanosarcina mazei* is a nonhalophilic methanogenic archaeon that can adapt to 800 mM NaCl. Microarray studies were performed to analyze the effect of elevated salinities on the regulation of gene expression in this microorganism. Over 80 genes of different functional categories were found to be more strongly expressed at high salinities (Pflüger et al. 2007). Amongst these genes there were phosphate-transporting genes. With the help of PPK, the Pi taken up by the Pst system under high salt conditions might be converted to polyP, which can serve as a phosphate or energy storage or play regulatory roles (Pflüger et al. 2007). Nevertheless, the mechanism for salt adaptation involving polyP in *M. mazei* remains to be demonstrated.

Role of polyP in metal resistance in extremophiles

A. ferrooxidans ATCC 23270 can survive high copper concentrations by having in its genome at least ten genes that are possibly related to copper homeostasis.

A. ferrooxidans ATCC 53993 has the same copper-resistance determinants than those of strain ATCC 23270, but in addition it has an exclusive genomic island with extra copper-resistance determinants which confer it a higher metal resistance (Orellana and Jerez 2011). As seen in Fig. 5, these include three genes coding for putative ATPases related to the transport of copper ($copA1_{Af}$, $copA2_{Af}$, and $copB_{Af}$), three genes related to a system of the RND family, involved in the extraction of copper from the cell by using the proton-motive force ($cusA_{Af}$, $cusB_{Af}$, $cusC_{Af}$), and two genes coding for periplasmic chaperones for this metal ($cusF_{Af}$ and $copC_{Af}$) (Navarro et al. 2009). The expression of most of these open reading frames (ORFs) was studied by real time RT-PCR (qRT-PCR) using *A. ferrooxidans* cells that were adapted to grow in the presence of high concentrations of $CuSO_4$. These *A. ferrooxidans* copper-resistance determinants were found to be upregulated when this bacterium was exposed to $CuSO_4$ in the range of 5–25 mM and conferred to *E. coli* a greater resistance to copper compared to wild-type cells, supporting their functionality (Navarro et al. 2009). The proteins regulating the copper-resistance response in *A. ferrooxidans* are yet to be described.

When external Cu concentration increases, all of the Cu-resistance determinants from *A. ferrooxidans* are expressed at higher levels to eliminate Cu from the periplasm or cytoplasm of the cells (Fig. 5). This requires high levels of ATP to activate the metal efflux ATPases. The cus system incorporates a proton for each copper eliminated, and therefore it could acidify the cytoplasm.

A relationship between polyP metabolism and heavy metal resistance has been proposed for cadmium detoxification in *E. coli* (Keasling 1997). Based on these results, a polyP-dependent system for Cu resistance has been proposed for *A. ferrooxidans* (Alvarez and Jerez 2004) and *S. metallicus* (Remonsellez et al. 2006). PolyP is synthesized by PPK in *A. ferrooxidans* or other bacteria (or by a yet unknown equivalent archaeal enzyme) by using ATP (Fig. 5). However, in excess of ADP generated by the use of cellular ATP, the reverse reaction of PPK synthesizes more ATP from polyP. In this way, the reserve polyP would also be supplying energy to the metal detoxifying systems.

In the presence of Cu, *A. ferrooxidans* and *S. metallicus* cells showed a rapid decrease in polyP levels with a concomitant increase in exopolyphosphatase activity and a stimulation of phosphate efflux. This may be the result of the hydrolysis of polyP by PPX to remove Cu–phosphate complexes formed. The metal–phosphate complexes would be pumped out to the periplasmic space (*A. ferrooxidans*) (Alvarez and Jerez 2004) or to the exterior (*S. metallicus*) (Remonsellez et al. 2006) by means of carriers of inorganic phosphate such as Pit (for phosphate inorganic transport) as proposed for *E. coli*, where the carrier is located in the

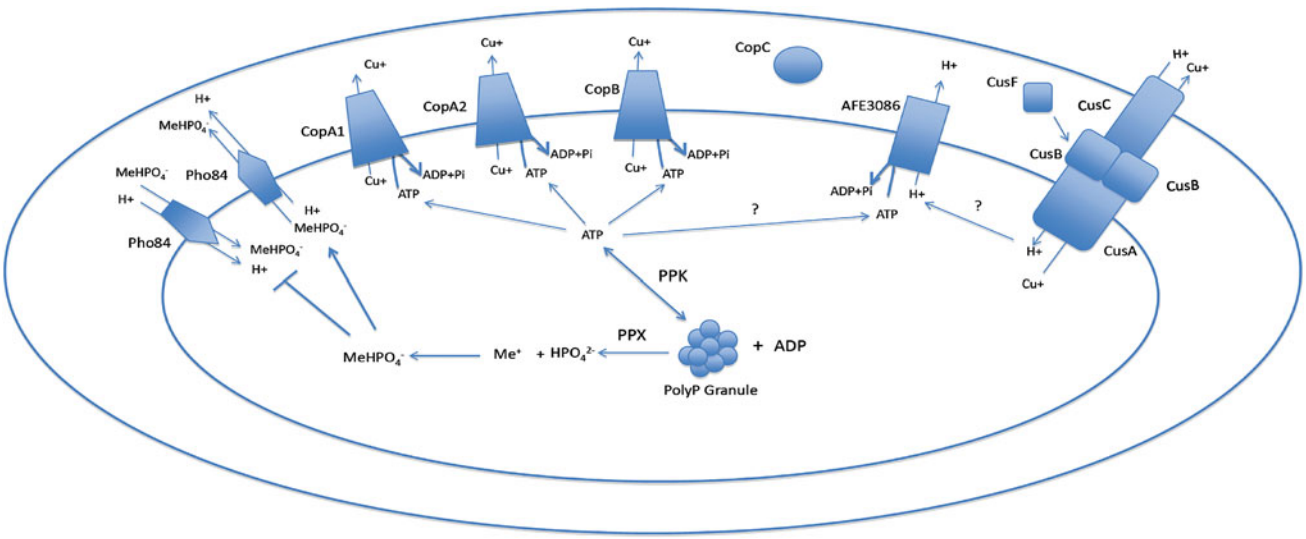

Fig. 5 Working model on the role of polyP in metal resistance in acidophilic extremophiles. The Cu-resistance determinants from *A. ferrooxidans* ATCC 23270 whose expression has been demonstrated to increase in the presence of Cu (CopA1, CopA2, CopC, CopB, CusF, CusA, CusB, CusC) are indicated. AFE 3086 corresponds to a putative plasma membrane proton-efflux P-type ATPase. PPK is the polyphosphate kinase enzyme that synthesizes polyP and PPX is the exopolyphosphatase that hydrolyzes the polymer. Pho84 is a putative phosphate transporter that *A. ferrooxidans* and acidophilic archaeons could use instead of the lacking Pit systems. *Sulfolobales* and other archaea contain only the Cu-resistance determinants CopA, CopT, CopM. PPK is not known in Crenarchaea and PPX is the exopolyphosphatase that hydrolyzes the polymer. This model is based, in part, on the results of Van Veen et al. (1994), Keasling (1997), Outten et al. (2001), Cardona et al. (2002), Alvarez and Jerez (2004), Remonsellez et al. (2006), Ettema et al. (2006), Auernik et al. (2008) and Navarro et al. (2009)

internal membrane of the bacterium. *A. ferrooxidans*, *S. solfataricus*, *S. acidocaldarius*, *S. tokodaii* and *M. sedula* do not show putative genes for a Pit system in their genomes. However, they have one or more ORFs coding for a putative yeast Pho84 that could act as a phosphate transporter. It has been demonstrated that in *S. cerevisiae* Pho84 can transport $MeHPO_4^-$ complexes only in acidic conditions (Fristedt et al. 1999).

With respect to the polyP-dependent mechanism proposed for Cu detoxification in *Sulfolobales*, it would be similar to that shown in Fig. 5 for *A. ferrooxidans*, except that no periplasmic and outer membrane components would be present and only one or two copA-type ATPases that have been described in the archaeons would be functioning to remove copper from the cytoplasm (Ettema et al. 2006). In addition, copper-responsive regulators that appear unique to the archaeal domain have been described recently (Villafane et al. 2011). An ORF with similarity to the PPX previously characterized in *S. solfataricus* (Cardona et al. 2002) has been reported in *M. sedula*. Furthermore, as with other acidophiles (Alvarez and Jerez 2004; Remonsellez et al. 2006), when using the *S. cerevisiae* Pho84 sequence and the top Pho84-like sequence in *A. ferrooxidans* as queries, four hits with similarities of 30–32 % to the major facilitator superfamily of substrate transporters were found in *M. sedula* (Auernik et al. 2007). Although it should be confirmed experimentally, the presence of these putative genes and abundant polyP granules (Fig. 1) also suggests the possible existence of a polyP-based Cu-resistance system in *M. sedula*.

These proposed mechanisms for metal resistance need to be proven, but they may be eventually functional in all polyP-accumulating biomining microorganisms and other extremophiles. To further support whether the capacity to accumulate and/or degrade polyP is related to copper resistance in *Sulfolobus* species, a *S. solfataricus* recombinant strain unable to accumulate polyP was generated (Orell, Albers and Jerez, unpublished results). This was achieved by the overexpression of the endogenous PPX and we were able to eliminate most of the cellular polyP (>98 %) in this *S. solfataricus* recombinant strain. Furthermore, we determined a decrease of 3-fold of its copper MIC value in comparison to the wild-type strain, which presented normal polyP levels. To our understanding, the augmented copper sensitivity shown by the polyP-deficient *S. solfataricus* strain corresponds to a direct experimental evidence for the involvement of polyP in copper resistance (Orell and Jerez, unpublished results). The lack of polyP would produce both an energy deficit and a state of increased stress which would most likely make the cells more sensitive to damaging conditions such as exposure to copper or other harmful stimuli. Further assays are being performed in order to reveal this latest hypothesis.

The presence in acidophiles of genes with similarity to most of the Cu-resistance determinants contained in neutrophilic microorganisms does not completely explain the much higher metal resistance of some of the acidophiles. As already mentioned, the presence of extra copies of these genes may give them an additional capacity to better resist the metal. Nevertheless, it is possible that multiple systems may contribute simultaneously to provide synergistic Cu resistance (Orell et al. 2010; Orellana and Jerez 2011).

A connection between pH homeostasis and metals homeostasis is not clear yet. However, it is a known fact that polyP has a high buffering capacity, being able to neutralize protons and metals that could be incorporated into the cytoplasm (Rao et al. 2009). The role we are proposing for polyP in eliminating Cu in acidophilic bacteria may be connected with its capacity to neutralize part of the protons generated by the Cus-like systems when Cu is being extruded to the outside of the cells in the acidophilic microorganisms (Fig. 5).

A main strategy for bacterial pH homeostasis is the use of transporters that catalyse active proton transport, such as proton-coupled ATPases. Interestingly, these proteins are constitutively expressed in extremophiles, so these microorganisms are ready for sudden pH changes (Krulwich et al. 2011). *A. ferrooxidans* has an inner-membrane proton pump or F1F0-ATPase to generate ATP by using the favorably proton-motive force generated by its proton gradient of several pH units (Wakai et al. 2005). The activity of this protein complex could in theory acidify the bacterial cytoplasm. However, the aerobic energetic metabolism of *A. ferrooxidans* uses oxygen as the final electron acceptor to form water, thus consuming any possible excess of cytoplasmic protons.

Unpublished results of BLAST analysis using the genome of *A. ferrooxidans* ATCC 23270 indicated the presence of ORF AFE_3086 whose deduced genetic product has a high identity with putative plasma membrane proton-efflux P-type ATPases. In addition, orthologs of AFE_3086 are present in different acidophilic species such as *A. caldus*, *Thiomonas* sp. 3As, *Leptospirillum ferrooxidans*, *M. sedula*, *S. islandicus*, *Ferroplasma acidarmanus* amongst others. Therefore, these putative proton-ATPases could be important to maintain intracellular pH homeostasis during the detoxification of copper or other metals (Fig. 5).

Intracellular polyP accumulation in response to an acid environment has been reported in several non-extremophilic environmental bacteria such as *Burkholderia cepacia* (Mullan et al. 2002) and in the yeast *Candida humicola* (McGrath and Quinn 2000). Therefore, this phenomenon may be a widespread microbial response to low external pH values.

In summary, although current knowledge of extremophiles is scarce, the actual findings indicate that inorganic

polyphosphates may play an important role in metal resistance, salt tolerance, oxidative stress adaptation, temperature tolerance and other conditions found under environmental stress situations which extremophilic microorganisms are confronted with. It is envisaged that additional efforts need to be taken in terms of providing (1) evidence for the direct role of polyP in the proposed metal resistance model, (2) regulatory insights on polyP metabolism, (3) a description of the crenarchaeal polyP synthase activity and (4) knowledge about signaling transduction events connecting the external stimuli with polyP responses. These advances may lead in turn to have genetically modified extremophiles more fit for their application in industrial biotechnological processes.

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