Global effect of the lack of inorganic polyphosphate in the extremophilic archaeon Sulfolobus solfataricus: A proteomic approach

Daniela F. Sotoa, Alejandra Recaldea, Alvaro Orellb, Sonja-Verena Albersc, Alberto Paradeld, Claudio A. Navarroa, Carlos A. Jerez*,

* Laboratory of Molecular Microbiology and Biotechnology, Department of Biology, Faculty of Sciences, University of Chile
b Max Planck Institute for Terrestrial Microbiology, Marburg, Germany
c Molecular Biology of Archaea, Institute of Biology II-Microbiology, University of Freiburg, Freiburg, Germany
d Proteomic Service, CNB-CSIC, Madrid, Spain

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ABSTRACT

Inorganic polyphosphates (polyP) are present in all living cells and several important functions have been described for them. They are involved in the response to stress conditions, such as nutrient depletion, oxidative stress and toxic metals amongst others. A recombinant strain of Sulfolobus solfataricus unable to accumulate polyP was designed by the overexpression of its endogenous ppx gene. The overall impact of the lack of polyP on this S. solfataricus polyP (−) strain was analyzed by using quantitative proteomics (isotope-coded protein label, ICPL). Stress-related proteins, such as peroxiredoxins and heat shock proteins, proteins involved in metabolism and several others were produced at higher levels in the ppx expression strain. The polyP deficient strain showed an increased copper sensitivity and an earlier transcriptional up-regulation of copA gene coding for the P-type copper-exporting ATPase. This implies a complementary function of both copper resistance systems. These results strongly suggests that the lack of polyP makes this hyperthermophilic archaeon more sensitive to toxic conditions, such as an exposure to metals or other harmful stimuli, emphasizing the importance of this inorganic phosphate polymers in the adaptations to live in the environmental conditions in which thermoacidophilic archaea thrive.

Significance: Inorganic polyphosphate (polyP) are ubiquitous molecules with many functions in living organisms. Few studies related to these polymers have been made in archaea. The construction of a polyP deficient recombinant strain of Sulfolobus solfataricus allowed the study of the global changes in the proteome of this thermoacidophilic archaeon in the absence of polyP compared with the wild type strain. The results obtained using quantitative proteomics suggest an important participation of polyP in the oxidative stress response of the cells and as having a possible metabolic role in the cell, as previously described in bacteria. The polyP deficient strain also showed an increased copper sensitivity and an earlier transcriptional up-regulation of copA, implying a complementary role of both copper resistance systems.

1. Introduction

Inorganic polyphosphate (polyP) is a linear polymer of hundreds to thousands of orthophosphate residues linked by high energy phosphoanhydride bonds [1]. PolyP is present in all kinds of organisms: prokaryote and eukaryote and its origin seems to be pre-biotic. In Bacteria and Euryarchaeota, these inorganic polymers are synthetized mainly by the enzyme polyphosphate kinase (PPK) from the terminal phosphate of an ATP molecule in a reversible reaction.

\[
\text{(polyP)}_n \text{ATP} \xrightleftharpoons{\text{PPK}} \text{(polyP)}_{n+1} + \text{ADP}
\]

In Bacteria and Crenarchaeota, the enzyme degrading polyP is the exopolypolypophatase (PPX), that hydrolyses the polymer liberating inorganic phosphate (Pi) [1,2].

\[
\text{(polyP)}_{n+1} \xrightarrow{\text{PPX}} \text{(polyP)}_n + \text{Pi}
\]

PolyP is involved in a variety of functions in the cell, such as a phosphate reservoir and a substitute for ATP in some kinase reactions. In addition, it is involved in adaptations to different stress conditions, such as virulence, biofilm formation and the detoxification of heavy metals in the cell by chelation [1,2]. It was recently reported that polyP may also act as a primordial inorganic protein chaperone in Escherichia coli.
coli, preventing proteins damage due to reactive oxygen species (ROS) [3].

*Sulfolobus solfataricus* is a model thermoacidophilic archaean that lives in a pH range between 2 and 4, and temperatures from 60 to 92 °C and can grow utilizing different carbon sources [4]. One of the advantages of working with this organism is that some tools for its genetic manipulation are available [4,5].

Contrary to other archaea, such as *Sulfolobus metallicus*, *S. solfataricus* does not accumulate polyP in the form of visible granules [6]. The enzyme involved in the synthesis of the polymer is currently unknown [3].

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There are few studies related to polyP and its functions in archaea [6,8]. To further study the different roles polyP may have in this domain of life, a global quantitative proteomic analysis of a *Sulfolobus solfataricus* strain deficient in polyP (M16-PPX) was carried out. This mutant strain was generated by the overproduction of its own PPX in the presence of β-arabinose.

2. Materials and methods

2.1. Strains and growth conditions

*S. solfataricus* strains M16 (pyrEF mutant, auxotroph for uracil) [9] and M16-PPX (complemented for pyrEF and containing a copy of *S. solfataricus* ppx gene inducible by β-arabinose) were used in this study. Both strains were grown at 75 °C with shaking at 120 rpm in Brock Medium (1.3 g/L (NH₄)₂SO₄, 0.28 g/L KH₂PO₄, 0.25 g/L MgCl₂ × 7 H₂O, 0.07 g/L CaCl₂ × 2 H₂O, 0.02 g/L FeCl₂ × 4 H₂O, 1.8 mg/L MnCl₂ × 4 H₂O, 4.5 mg/L Na₂B₄O₇ × 10 H₂O, 0.22 mg/L ZnSO₄ × 7 H₂O, 0.06 mg/L CuCl₂ × 2 H₂O, 0.03 mg/L Na₂MoO₄ × 2 H₂O, 0.03 mg/L Na₂S × 2 H₂O and 0.01 mg/L CaCl₂ × 6 H₂O) pH 3 and supplemented with 0.1% (p/v) N-Z amine and 0.01 mg/mL uracil except for 2-3 days until 0.5 OD₆₀₀ nm was reached. To select non-auxotrophic cells for uracil, aliquots from the previous culture where used to inoculate fresh Brock medium with only N-Z amine as supplement. Cells grown in this medium were induced in the presence of β-arabinose to verify the overproduction of PPX. Finally, aliquots of the cells suspension were stored in 20% glycerol at −80 °C.

2.2. Transformation of *S. solfataricus* M16 (pyrEF, auxotroph for uracil) into the expression vector pMZ1

Transformation of cells was done by electroporation. Fifty milliliter of *S. solfataricus* M16 (uracil auxotroph) grown until exponential phase (0.3 OD₆₀₀ nm) were centrifuged for 30 min at 4 °C, washed several times with 20 mM saccharose, pH5.6 to eliminate salts. Finally, cells were resuspended in the same saccharose solution at 10¹⁰ cells/mL.

Fifty microliter aliquots of the cells suspension were mixed with 150, 300 or 450 ng of plasmid pJ0503-r_ppxS.so and transferred to BioRad electroporation cuvettes, where electroporation was carried out under the following conditions: 1.5 kV, 25 μF and 400 Ω. Next, the cells suspension was diluted into 1 ml of fresh Brock medium supplemented with 0.1% N-Z amine and were further incubated for 1 h at 75 °C with vigorous agitation. Cells were then transferred to 50 ml of Brock medium supplemented with 0.1% N-Z-amine and 0.01 mg/mL uracil and grown for 2-3 days until 0.5 OD₆₀₀ nm was reached. To select non-auxotrophic cells for uracil, aliquots from the previous culture where used to inoculate fresh Brock medium with only N-Z amine as supplement. Cells grown in this medium were induced in the presence of β-arabinose to verify the overproduction of PPX. Finally, aliquots of the cells suspension were stored in 20% glycerol at −80 °C.

2.3. Production and purification of recombinant PPX (rPPX)

To induce the production of rPPX, *S. solfataricus* M16-PPX cells were grown up to 0.4 OD₆₀₀ nm, followed by the addition of 0.2% β-arabinose and further incubation for 4 h. Cells were then collected by centrifugation and resuspended in sonication buffer (50 mM Tris–HCl pH 8.5, 10 mM EDTA) containing phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor (100 μg/ml). Cells were then disrupted by sonication on ice (Ultrasonic Liquid Processor, SONICATOR®), using 9 cycles of 20 s pulses with 30 s of pauses in between. The lysate was centrifuged at 100,000 × g for 1 h at 4 °C and 5 ml of the supernatant was applied to a column containing 1 ml of his-NTA resin with NioSa (Sigma-Aldrich). The column was washed three times with 800 μl of wash buffer (50 mM Hepes pH 8.5, 50 mM KCl and 30 mM Imidazole). Finally, rPPX was eluted in 3 fractions of 400 μl elution buffer (50 mM HEPES pH 7.0, 50 mM KCl and 250 mM Imidazole). Fractions of 0.5 ml were collected and analyzed by SDS-PAGE and Western blot by using rabbit monoclonal antibodies against His tag.

2.4. PolyP extraction from *S. solfataricus* cells

Cells from a 5 ml culture were collected by centrifugation for 5 min at 4500 × g. The pellet was resuspended in 0.3 ml of 4 M guanidine isothiocyanate (GITC), 50 mM Tris–HCl pH 7.0 prewarmed at 95 °C as previously described [1,6]. The suspension was agitated in vortex and heated at 95 °C for 3 min. Twenty μl of each sample was saved for protein quantification with Coomassie Plus Protein Assay Reagent (PIERCE). 30 μl of 10% SDS was added to each sample and then they were heated again at 95 °C for 5 more min. 300 μl of ethanol (100%) was used to over-produce PPX in *S. solfataricus*. This vector integrates into the *S. solfataricus* genome and contains the pyrEF gene as a selection marker. First, pMZ1-ppxS.so was digested with 4 U of BlnI and EagI, in NEB3 buffer 1 × (New England Biolabs) for 3 h at 37 °C and the product obtained was purified after its separation by agarose gel electrophoresis. Vector pMJ0503 was digested with the same restriction enzymes under identical conditions. Forty ng of the digestion product and 5 ng of digested pMJ0503 vector were mixed with T4 DNA ligase buffer 1 × and 5 U of T4 DNA ligase. Ligation reaction was carried out for 16 h at 4 °C. Five μl of the ligation mix was used to transform *E. coli* Stable4 (Stratagene®), according to the manufacturer’s instructions. Positive clones were selected with ampicillin by employing colony PCR and the primers designed for ppxS.so. Finally, pJ0503-r_ppxS.so was sequenced for DNA identity verification.

2.2.2. Cloning of recombinant ppxS.so gene (r_ppxS.so) into the expression vector pMZI0503

The binary vector pMJ0503 (for *E. coli* and *S. solfataricus*) [5,10] was used to over-produce PPX in *S. solfataricus*. This vector integrates into the *S. solfataricus* genome and contains the pyrEF gene as a selection marker. First, pMZ1-ppxS.so was digested with 4 U of BlnI and EagI, in NEB3 buffer 1 × (New England Biolabs) for 3 h at 37 °C and the product obtained was purified after its separation by agarose gel electrophoresis. Vector pMJ0503 was digested with the same restriction enzymes under identical conditions. Forty ng of the digestion product and 5 ng of digested pMJ0503 vector were mixed with T4 DNA ligase buffer 1 × and 5 U of T4 DNA ligase. Ligation reaction was carried out for 16 h at 4 °C. Five μl of the ligation mix was used to transform *E. coli* Stable4 (Stratagene®), according to the manufacturer’s instructions. Positive clones were selected with ampicillin by employing colony PCR and the primers designed for ppxS.so. Finally, pJ0503-r_ppxS.so was sequenced for DNA identity verification.

2.2.3. Transformation of *S. solfataricus* M16 with pJ0503-r_ppxS.so

Transformation of cells was done by electroporation. Fifty milliliter of *S. solfataricus* M16 (uracil auxotroph) grown until exponential phase (0.3 OD₆₀₀ nm) were centrifuged for 30 min at 4 °C. Cells obtained were washed several times with 20 mM saccharose, pH5.6 to eliminate salts. Finally, cells were resuspended in the same saccharose solution at 10¹⁰ cells/mL.

Fifty microliter aliquots of the cells suspension were mixed with 150, 300 or 450 ng of plasmid pJ0503-r_ppxS.so and transferred to BioRad electroporation cuvettes, where electroporation was carried out under the following conditions: 1.5 kV, 25 μF and 400 Ω. Next, the cells suspension was diluted into 1 ml of fresh Brock medium supplemented with 0.1% N-Z amine and were further incubated for 1 h at 75 °C with vigorous agitation. Cells were then transferred to 50 ml of Brock medium supplemented with 0.1% N-Z-amine and 0.01 mg/mL uracil and grown for 2-3 days until 0.5 OD₆₀₀ nm was reached. To select non-auxotrophic cells for uracil, aliquots from the previous culture were used to inoculate fresh Brock medium with only N-Z amine as supplement. Cells grown in this medium were induced in the presence of β-arabinose to verify the overproduction of PPX. Finally, aliquots of the cells suspension were stored in 20% glycerol at −80 °C.
and 5 μL of silica (Glassmilk) was added and mixed by vortexing followed by heating at 95°C for 30 s. After 1 min centrifugation at 13,000 x g, the supernatant was removed and the glassmilk pellet containing polyP, DNA and RNA was resuspended in 200 μL of cool New Wash Buffer (5 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 50% ethanol). After centrifugation, glassmilk was finally resuspended in 100 μL of a solution containing 50 mM Tris-HCl pH 7.0, 5 mM MgCl₂, 5 μg/μL DNase and 5 μg/μL RNase and incubated for 30 min at 37°C. The pellet was washed twice with New Wash Buffer and polyP was recovered from the glassmilk after repeatedly vortexing it with 50 μL of water, heating at 95°C and centrifugation. Finally, a total of 100 μL of polyP-containing solution was obtained and frozen at −20°C for later quantification.

2.5. Enzymatic determination of polyP content

Thirty microliter of polyP containing solution was used for quantification. The polymers were subjected to acid hydrolysis with 2 N HCl (30 μL) for 30 min at 95°C to release inorganic phosphate (P_i). Quantification of total P_i was done with EnzChek Phosphate Assay kit (Invitrogen) following manufacturer’s instructions and expressed as nmol of P_i per mg of protein.

2.6. Total protein extracts preparation for proteomic analysis

After 24 h of growth in the absence of polyP, cells were harvested by centrifugation (7700 × g for 15 min). The cell pellets were washed three times with Brock’s medium and lysed by sonication as described in Section 2.3. The lysate was centrifuged at 9800 × g for 5 min to eliminate unbroken cells and cell debris. Total protein concentration in the cell-free extract was determined by using the Coomassie Plus Protein Assay Reagent (PIERCE).

Micrograms from each of three biological replicates were mixed to obtain a triplicate representative sample with a total of 150 μg of protein from each experimental condition. These samples were lyophilized for 48 h at −40°C and stored at −20°C until they were labeled with isotope-coded protein label (ICPL).

2.7. Protein digestion and ICPL-labeling

The ICPL reagent protocol was optimized for labeling 100 μg of each individual sample per experiment. Thus, 100 μg of total protein extracts was individually dissolved in 8 M urea, 25 mM ammonium bicarbonate, reduced with 10 mM dithiothreitol and alkylated with 50 mM iodoacetamide. The urea concentration was reduced to 2 M with 25 mM ammonium bicarbonate and the sample was digested overnight at 37°C with trypsin (Roche Diagnostics GmbH, Mannheim, Germany) in a sample:protease ratio of 25:1. Before ICPL-labeling, salts and urea were removed by using high-capacity OMIX C18 tips (Varian, Palo Alto, CA). Sample labeling with the light and heavy versions of the ICPL reagent (Serva Electrophoresis, Heidelberg, Germany) was performed at the peptide level according to the manufacturer’s instructions.

2.8. 2D-nano LC ESI-MS/MS analysis

After ICPL-labeling samples were combined (200 μg per experiment) and dissolved in 100 μL of 10 mM NH₄OH in water, pH 9.5 and fractionated in a wide-pH range 5 μm particle size, 100 × 2.1 mm reversed phase XBridge column (Waters, Wexford, Ireland) using a Knauer Smartline HPLC system. Conditions for gradient elution, flow rate, injection volumes, number of HPLC fractions and second dimension of the 2D-nano LC ESI-MS/MS analysis were carried out as previously reported [11].

2.9. Protein identification and quantitative analyses

MS and MS/MS data obtained for individual HPLC fractions were merged using the Analysis Combiner tool and subsequently processed as a single experiment using DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany). In most cases, an accuracy of ± 0.1-0.2 Da was found both for MS and MS/MS spectra. For protein identification, MS/MS spectra (in the form of Mascot generic files) were searched against the S. solfataricus P2 UniprotKB forward-reversed database. (http://www.uniprot.org). Sequence reversal was done using the program DBToolkit v4.1.5. Database searches were done using a licensed version of Mascot v.2.2.04 (www.matrixscience.com; Matrix Science, London, UK). Search parameters were as previously described [12]. FDR ≤ 5% for peptide identification were manually assessed as follows: after database searching, peptide matches were ranked according to their Mascot scores. This list contains peptide sequences matching either forward or reversed database sequences. Then, a subset containing 5% of peptides matching the reversed sequences was extracted.

Qualitative and quantitative analyses were performed by using the software WARP-LC 1.1 (Bruker Daltonics, Bremen, Germany). After peptide identification, the software calculates the extracted ion chromatogram for the putative ICPL-labeled pair according to: (a) the mass shift defined by the labeling reagent, (b) a mass tolerance of 0.5 Da, and (c) a retention time tolerance of 40 s. Relative ratios between light and heavy ICPL-labeled peptides were calculated based on the intensity signals of their corresponding monoisotopic peaks, and according to these individual peptide ratios the software calculates the protein ratio.

2.10. Total RNA extraction from S. solfataricus and cDNA synthesis

To study the expression of genes of interest, cells were grown until they reached exponential growth phase (0.3 OD_600 nm) and were supplemented with 0.2% α-D-glucose. After 3 h of induction, 1 mM CuSO_4 was added and cells were collected (10 mg wet weight) at different times and lysed as previously described [13]. RNA was extracted by using TRIzol (Invitrogen*) as described by the manufacturer. Remaining DNA was eliminated by adding 40 U of TURBO DNA-free DNase (Ambion*) following manufacturer’s instructions. 0.8 μg of total RNA was reverse transcribed for cDNA synthesis. The reaction was performed by using ImProm-II (Promega) reverse transcriptase, 0.5 μg of random hexamers (Promega) and 3 mM MgCl₂ for 1 h at 42°C. Three biological replicates were used for each experimental condition.

2.11. Primer design and real-time RT-PCR

Primers for qRT-PCR were designed by using the annotated genome of S. solfataricus P2 and Primer3 Software, and were synthesized by Invitrogen. To check primer specific annealing and optimal melting temperature, PCR reactions were carried out with Taq DNA polymerase from Promega following manufacturer’s instructions and the products were separated by gel electrophoresis (1% w/v agarose). Gene expression was analyzed with Corbett Rotor Gene 6000 system (Corbett Research®), using 7.5 μL QuantiFast SYBR Green (Qiagen®) master mix, 1 μL of primer and 1 μL of a 1:20 dilution of the cDNA. rps2P was selected as housekeeping gene and Cq values of each transcript of interest were standardized to the Cq value of the rps2P gene. At least 2 technical replicates per qRT-PCR reaction were performed.

The efficiency of each primer pair was calculated from the average slope of a linear regression curve, which resulted from qPCRs using a 10-fold dilution series (10 pg–10 ng) of S. solfataricus chromosomal DNA as template. Efficiencies between 90 and 110% were used. Cq values (quantification cycle) were automatically determined by Real-Time Rotor-gene 6000 PCR software (Corbett Life Sciences/Quiangen company) after 40 cycles. Cq values of each transcript of interest were standardized to the Cq value of the rps2P gene. At least 3 biological replicates of each assessed condition and 2 technical replicates per
qPCR reaction were performed.

2.12. Statistical analysis

Data obtained was subjected to analysis of variance (ANOVA) and a Bonferroni’s test.

3. Results and discussion

3.1. M16-PPX strain overproduces PPX enzyme and lacks polyP

Strains M16 and M16-PPX were grown in the presence of either 0.1% glucose or 0.2% D-arabinose until the early stationary phase was reached. As shown in Fig. 1, polyP levels in M16 and M16-PPX were very similar when both strains were grown in the presence of glucose. However, in the presence of D-arabinose polyP levels were not detectable in M16-PPX. On the other hand, polyP levels in M16 remained the same when grown either in glucose or D-arabinose.

To determine the time it takes PPX to deplete polyP from the cells, S. solfataricus M16-PPX was grown until early exponential phase (0.3 OD_{600} nm) and was induced in the presence of D-arabinose. PolyP levels were measured at different times after induction. After 2.5–3 h, the polyP content in M16-PPX strain was not detectable by the method described in Section 2.5 (data not shown).

3.2. Quantitative proteomics

For proteomics analysis, proteins were extracted from S. solfataricus M16-PPX and the control M16 strain, both induced in the presence of D-arabinose for 24 h.

Fifty three proteins changed their levels in the polyP (−) construction. Twenty-nine proteins were up-regulated and 24 down-regulated. Metabolism was the functional category that included most of the changes in protein abundance in cells lacking polyP (Fig. 2). Thirty-four of them (64.2%) changed their levels in this functional category. “Energy production and conversion” was the subcategory with the greatest number of variations. This agrees with polyP being a source of phosphate for many enzymes, as previously described in bacteria [1].

“Information storage and processing” corresponded to the general category with the second number of changes (13 proteins or 24.5% of total). Meanwhile, 4 (7.5%) proteins of unknown function changed their levels in the absence of polyP.

3.2.1. Changes in carbohydrates and lipids transport and metabolism, and energy production and conversion related proteins

Amongst the up-regulated proteins (Table 1) there was an alpha amylase (SSO0988). This enzyme degrades glycogen and releases glucose to the intracellular medium to be incorporated into the energetic pathways (see Fig. 3). It was also reported that glycogen is a polysaccharide reservoir in thermoacidophilic archaea [14].

In the “Energy production and conversion” category, two subunits of the carbon monoxide dehydrogenase enzyme (small chain SSO2433 and large chain SSO2091) were overproduced (Table 1). This enzyme catalyzes the conversion of CO to CO2, and can also act as an acetyl-CoA synthase [15] generating acetyl-CoA which can be integrated into the TCA cycle (Fig. 3, in “other reactions of interest”).

Regarding the respiratory chain, a rise in the levels of an NADH dehydrogenase D subunit (SSO0324) was determined (Table 1) (bottom part of Fig. 3). The respiratory NADH dehydrogenase is an essential component of many bacterial respiratory chains. In S. solfataricus there is an activity apparently derived from a cytoplasmic flavin-containing NADH oxidase [16]. It also contains genes encoding for an NADH dehydrogenase core (SSO0665, SSO0322-0329) forming a nuoBCDHIL operon that would be partially equivalent to the one present in E. coli. It was described that the transcription of nuoBCDHIL operon increased after thermic stress in S. solfataricus [17]. Depletion of NADH levels is a
desirable situation to alleviate oxidative stress in microorganisms [18], since its passage through the respiratory chain generates ROS. Electron transfer flavoprotein (SSO2817) was also found up-regulated (not shown in Fig. 3).

In the “lipid transport and metabolism” category (Table 1), the acetyl-CoA dehydrogenase related protein (Acd-like1) increased its levels (SSO1156, not included in Fig. 3). This situation was previously reported in the heat shock response of S. solfataricus by Tacholjian and Kelly [17]. Because these transporters consume ATP to internalize sugars, a down regulation of these proteins could imply enough glucose generation by glycogen hydrolysis.

Secondary metabolites biosynthesis, transport and catabolism
Q97UR3 SSO2939 Maleate cis-trans isomerase, probable (MaiA) 116.57 6.81 4 1.64
Q97VU9 SSO2508 Acetyl-CoA acetyltransferase (Acetoacetyl-CoA thiolase) (AcAB-6) 44.48 23.68 2 5.95
Q97V9 SSO2508 Acetyl-CoA c-acetyltransferase (Acetoacetyl-CoA thiolase) (AcAB-6) 100.21 10.79 4 1.92

Table 1
More abundant proteins in Sulfolobus solfataricus M16-PPX, lacking polyP, compared to S. solfataricus M16.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>ORF</th>
<th>Function/similarity</th>
<th>Score (%)</th>
<th>Coverage (%)</th>
<th>Peptide number</th>
<th>Fold change (PolyP−/PolyP+)</th>
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<td>Q97ZD2 SSO0988</td>
<td>Alpha amylase</td>
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<td>Uncharacterized protein</td>
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<td>15.52</td>
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<td>Energy production and conversion</td>
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<tr>
<td>Q97W14 SSO2433</td>
<td>Carbon monoxide dehydrogenase, small chain</td>
<td>130.68</td>
<td>30.41</td>
<td>5</td>
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<td>Q7LX2 SSO2091</td>
<td>Carbon monoxide dehydrogenase, large chain</td>
<td>64.08</td>
<td>7.88</td>
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<td>2.23</td>
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<td>Q96O3 SSO324</td>
<td>NADH dehydrogenase subunit D (NuoD)</td>
<td>61.92</td>
<td>11.95</td>
<td>3</td>
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<td>Q97V19 SSO2817</td>
<td>Electron transfer flavoprotein, alpha and beta-subunit (EtfAB/fxAB)</td>
<td>130.08</td>
<td>18.03</td>
<td>8</td>
<td>1.75</td>
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<td>Lipid transport and metabolism</td>
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<td>Q97VY3 SSO1156</td>
<td>Acetyl-CoA dehydrogenase related protein (Acd-like1)</td>
<td>44.48</td>
<td>23.68</td>
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<tr>
<td>Q97V9 SSO2508</td>
<td>Acetyl-CoA c-acetyltransferase (Acetoacetyl-CoA thiolase) (AcAB-6)</td>
<td>100.21</td>
<td>10.79</td>
<td>4</td>
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<td>Q7LXG1 SSO1193</td>
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<td>Q98096 SSO0241</td>
<td>5-formaminoimidazole-4-carboxamide-1-(beta)-ribofuranosyl 5'-monophosphate synthetase</td>
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<td>Q9UXS24 SSO0629</td>
<td>Phosphoribosylformylglycinamid synthase, subunit PurL</td>
<td>108.93</td>
<td>17.21</td>
<td>4</td>
<td>1.95</td>
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<td>Gene expression and metabolism</td>
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<tr>
<td>Q97W7C1 SSO2303</td>
<td>Probable cobalt-precocin-6B (C15-methyltransferase</td>
<td>82.11</td>
<td>23.01</td>
<td>5</td>
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<td>P9599 SSO0661</td>
<td>Geranylderivative diphasphate synthase</td>
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<td>13.55</td>
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<td>8.77</td>
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<td>Acetolactate synthase large subunit homolog (IlvB6)</td>
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* Score in this table refers to protein score, according to Mascot search engine.
synthase (SSO1334) (Table 2, Fig. 3) may increase the TCA cycle activity. Malate synthase catalyzes the conversion of glyoxylate to malate with the consumption of Acetyl-CoA (Fig. 3). Therefore, a decrease in this protein would allow Acetyl-CoA to preferentially enter the TCA cycle instead of producing malate. Malate can also enter the TCA cycle, but generates lower reducing equivalents and ATP. Lower levels of malate synthase could lead to glyoxylate accumulation and subsequent ROS elimination during oxidative stress by a non-enzymatic decarboxylation of glyoxylate [18]. This ROS scavenger role has been described also for other keto-acids, such as pyruvate and α-ketoglutarate. This type of defense mechanisms seems to be of special importance for extremophilic microorganisms that do not have detoxifying mechanisms, such as catalases and glutathione [20]. Examples of these are *S. solfataricus* and ammonia oxidizing archaea [21].

Also, there were contradictory changes in proteins with similar functions. For example, Acetyl-CoA C-acetyltransferase isoforms AcaB-3 (SSO2062) and AcaB-4 (SSO2377) (Fig. 3) decreased their levels (Table 2). On the other hand, the isoform AcaB-6 (SSO2508) (Fig. 3) increased (Table 1). The activity of these enzymes is related to lipid metabolism, isoleucine degradation and carbon fixation cycle of 3-hydroxypropionate/4-hydroxybutirate. A similar discordance was also reported in the response of *S. solfataricus* to heat shock, were the genes acab-1,-3,-8,-10 decreased their expression, while acab-4,-5,-6 increased [17]. Each isoform is probably involved in different parts of the pathways, but further analysis would be necessary to clarify this point.

PolyP plays many roles in the cell. Perhaps, one of the most important is its ability to replace ATP in some reactions catalyzed by kinases, due to the presence of its high energy bonds analogous to those present in the ATP molecule [1]. It was also reported that the PPK enzyme can catalyze a reverse reaction, where polyP donates a phosphate group to ADP to convert it into ATP in *Escherichia coli* [1]. Although in crenarchaeotes the PPK enzyme has not been described yet, it is likely that an equivalent enzyme should synthesize polyP. Moreover, polyP also has been proposed to have a role in oxidative stress protection. It might act as an inorganic protein chaperone, and form complexes with metals, such as manganese, which can detoxify O$_{2}^-$ or iron, preventing the formation of ROS through the Fenton’s reaction [22]. Due to its aerobic metabolism and the presence of iron in the medium, *S. solfataricus* is constantly exposed to ROS generation. Thus, the lack of polyP should also affect its oxidative stress response.

### 3.2.2. Changes in Nucleotide transport and metabolism

In this category, the exopolypophatase (SSO1193) was found up-regulated (Table 1). This was expected since strain M16-PPX constructed here is overproducing its own rPPX to eliminate as much polyP as possible (Fig. 1).
3.2.3. Changes in Coenzyme transport and metabolism

In this group, an increased level of a probable cobalt-precorrin-6B C(15)-methyl transferase (SSO2303), a protein involved in vitamin B12 synthesis was identified (Table 1; not included in Fig. 3). This vitamin is a cofactor for many enzymes with a variety of activities, such as isomerases, methyl transferases and dehalogenases [23]. Additionally, an increase in the enzyme geranylgeranyl pyrophosphate synthase (SSO0061) was observed (Table 1, Fig. 3). A higher level of this enzyme would increase the concentration of geranyl-PP, geranylgeranyl-PP and farnesyl-PP. These three compounds are relevant for the synthesis of terpenoids and membrane lipids, and also the synthesis of ubiquinone, which are involved in the respiratory chain. They receive a cofactor for many enzymes with a variety of activities, such as isomerases, methyl transferases and dehalogenases [23]. Additionally, an increase in the enzyme geranylgeranyl pyrophosphate synthase (SSO0061) was observed (Table 1, Fig. 3). A higher level of this enzyme would increase the concentration of geranyl-PP, geranylgeranyl-PP and farnesyl-PP. These three compounds are relevant for the synthesis of terpenoids and membrane lipids, and also the synthesis of ubiquinone, which are involved in the respiratory chain. They receive a cofactor for many enzymes with a variety of activities, such as isomerases, methyl transferases and dehalogenases [23].

In this category an increase in the levels of serine-pyruvate aminotransferase (Agxt) (SSO2597) was detected (Table 1, Fig. 3). This enzyme was first described to participate in the non-phosphorylative pathway of serine. However, some studies point out that Agxt might participate in the phosphorylative pathway in *S. tokodaii*, a close member of Sulfolobales [26]. The increase in the enzymes related to serine biosynthesis (SSO0905 and SSO2597) not only would affect this particular process, but also other amino acids that can be obtained from it, such as glycine and threonine.

Lastly, another protein with an increased level in *S. solfataricus* cells lacking polyP was the large subunit of Acetolactate synthase (SSO3210). This enzyme was first described to participate in the non-phosphorylative pathway of serine. However, some studies point out that Agxt might participate in the phosphorylative pathway in *S. tokodaii*, a close member of Sulfolobales [26]. The increase in the enzymes related to serine biosynthesis (SSO0905 and SSO2597) not only would affect this particular process, but also other amino acids that can be obtained from it, such as glycine and threonine.

3.2.4. Changes in Amino acid transport and metabolism

In this category an increase in the levels of serine-pyruvate aminotransferase (Agxt) (SSO2597) was detected (Table 1, Fig. 3). This enzyme was first described to participate in the non-phosphorylative pathway of serine. However, some studies point out that Agxt might participate in the phosphorylative pathway in *S. tokodaii*, a close member of Sulfolobales [26]. The increase in the enzymes related to serine biosynthesis (SSO0905 and SSO2597) not only would affect this particular process, but also other amino acids that can be obtained from it, such as glycine and threonine.

Lastly, another protein with an increased level in *S. solfataricus* cells lacking polyP was the large subunit of Acetolactate synthase (SSO3210). This enzyme was first described to participate in the non-phosphorylative pathway of serine. However, some studies point out that Agxt might participate in the phosphorylative pathway in *S. tokodaii*, a close member of Sulfolobales [26]. The increase in the enzymes related to serine biosynthesis (SSO0905 and SSO2597) not only would affect this particular process, but also other amino acids that can be obtained from it, such as glycine and threonine.

Lastly, another protein with an increased level in *S. solfataricus* cells lacking polyP was the large subunit of Acetolactate synthase (SSO3210). This enzyme was first described to participate in the non-phosphorylative pathway of serine. However, some studies point out that Agxt might participate in the phosphorylative pathway in *S. tokodaii*, a close member of Sulfolobales [26]. The increase in the enzymes related to serine biosynthesis (SSO0905 and SSO2597) not only would affect this particular process, but also other amino acids that can be obtained from it, such as glycine and threonine.

3.2.5. Changes in Post transcriptional modification, protein turnover and chaperones

In this category, two peroxiredoxins increased their levels: Bcp-4 (SSO2613) and Bcp-2 (SSO2121) (Table 1 and upper left part in Fig. 3).
Both proteins play a central role in protecting *S. solfataricus* from oxidative stress and damage, coupled to the TCA cycle and the respiratory chain [28]. They convert H$_2$O$_2$ into H$_2$O and O$_2$, regenerate NAD content in the cell and maintain redox balance. The increase in peroxiredoxins has been previously reported in *S. solfataricus* in response to H$_2$O$_2$ [29] and in cells of *Pseudomonas* sp. B4 lacking polyP [25]. These findings are in agreement with the role of polyP in protection of cells against oxidative damage [22], and the recently described role of polyP as an inorganic chaperone to avoid protein denaturation in response to reactive oxygen species [3].

3.2.6. Changes in other categories

Interestingly, in the “cell wall, membrane and envelope biogenesis” category, the sulfolipid biosynthesis protein (SqdB) was down-regulated (Table 2, not included in Fig. 3). This enzyme seems to catalyze the N-glycosylation of some membrane components in Crenarchaeota [30]. Finally, several proteins of unknown function were found up and down-regulated (Tables 1 and 2). Their function and relationship to polyP metabolism remains unidentified. In general, an increment in the glycolytic pathway over the glycogenic pathway was observed in the absence of polyP. Under these conditions, the cell should reduce waste of energy.

More studies are needed to determine the real metabolic state of the cells lacking polyP, since enzyme activities depend not only in their concentration, but also in their induction or inhibition by other factors and in other levels of regulation [31]. Protein phosphorylation/dephosphorylation, is a process that might regulate many enzymes and transcription factor proteins in *S. solfataricus* [31–33], and could also be affected due the lack of polyP [1].

3.3. Possible role of polyP in copper resistance in *S. solfataricus*

Copper is able to generate ROS by the Fenton reaction [18,34] and it was previously shown that polyP plays a role in copper resistance in archaea [8]. Therefore, it was of interest to assess metal susceptibility of *S. solfataricus* strain M16-PPX. For this, the minimum inhibitory concentration (MIC) of copper was determined in M16-PPX, polyP (−). Cells were grown in Brock medium supplemented with either 0.2% D-arabinose or 0.1% glucose, with different CuSO$_4$ concentrations for 92 h until late exponential phase. MIC was defined as the concentration of copper capable of 50% inhibition of growth. As a control, a recombinant *S. solfataricus* strain overproducing a protein not related to copper resistance was used (data not shown).

As seen in Fig. 4A, M16-PPX grown in glucose has an estimated MIC value of 2 mM Cu. On the contrary, cells grown in α-arabinose, had a MIC value of only 0.75 mM Cu. This result clearly indicates the importance of polyP in copper resistance in *S. solfataricus*.

3.4. Effect of copper in the growth of *S. solfataricus* M16-PPX PolyP (−)

Cells were allowed to accumulate polyP until they reached an OD$_{600}$ nm of 0.25. They were then transferred to a fresh medium containing either 0.2% α-arabinose or 0.1% glucose as the control condition, and further incubated for 3 h to eliminate all detectable polyP present in the cells. CuSO$_4$ was then added to the medium at the indicated final concentrations and the OD$_{600}$ nm was registered in each case during 48 h. As shown in Fig. 4B, Cu affected preferentially cells lacking polyP.

Since *S. solfataricus* contains fewer canonical type of copper resistance mechanisms (apparently only two cop systems compared with some other microorganisms) [35], it is possible that the proposed polyP-mediated metal detoxification plays an important role in metal resistance, as suggested before [34,36].

3.5. CopA transcriptional levels in *S. solfataricus* M16 and M16-PPX exposed to copper

CopA is a P-type copper-exporting ATPase that along with CopM (a metal chaperone) and CopT (a transcriptional regulator) forms part of a mechanism for copper resistance in *S. solfataricus* [35]. As already mentioned, polyP seems to play an important role in copper resistance [6,34]. It was of interest therefore, to compare the copA transcriptional levels of both M16-PPX and M16 strains grown in the presence of copper.

Both M16 and M16-PPX *S. solfataricus* strains were grown until they reached an OD$_{600}$ nm of 0.3 followed by the induction of ppx in the presence of α-arabinose (0.2%) for 3 h. At this time copper was added to have 1 mM final concentration and total RNA was extracted at the times of incubation indicated in Fig. 5. Both kinds of cells (with and without polyP), showed an increased level of transcriptional expression of copA in the presence of copper until a maximum value was reached and declining thereafter. However, cells lacking polyP, had a maximum level of copA at 20 min, being much higher than that of M16 cells reaching their maximal copA levels after 30 min of incubation in the presence of copper. These results suggest that in the absence of polyP, CopA may have an important initial role in the response of *S. solfataricus* to copper.
Altogether, the results presented here support that polyP plays a variety of roles in the hyperthermophilic archaeon *S. solfataricus*, and the lack of this polymer triggers a variety of responses, suggesting a similar state to the one caused by oxidative stress.

Regarding copper resistance, as previously reported in the related archaea *S. metallicus*, *M. sedula* and bacteria, polyP may act as a counter ion, helping the expulsion of copper cations through PitA and possibly Pho84-like systems. Recently, a strain of *Sulfolobus solfataricus*, *PitA* and a higher copper resistance than the wild type (who has an intact *copA* gene) has been described [37]. This points out the importance of this phosphate transporter in copper resistance. Nevertheless further studies will be necessary to unravel the complexity of polyP functions in archaeal cells.

Conflict of interest
The authors declare no conflict of interest.

Transparency document
The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.02.024.

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