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**Research Article**

**Title: Bacterial microcompartment-directed polyphosphate kinase promotes stable polyphosphate accumulation in *E. coli***

Mingzhi Liang<sup>1,2</sup>, Stefanie Frank<sup>2</sup>, Heinrich Lünsdorf<sup>3</sup>, Martin J Warren<sup>2\*</sup>,  
Michael B Prentice<sup>1,4,5\*</sup>

<sup>1</sup>Department of Microbiology, University College Cork, Cork, Ireland

<sup>2</sup>School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK

<sup>3</sup>Central Facility for Microscopy, Helmholtz Center of Infection Research, Braunschweig, D-38124, Germany

<sup>4</sup>Department of Pathology, University College Cork, Cork, Ireland

<sup>5</sup>APC Microbiome Institute, University College Cork, Cork, Ireland

**Correspondence:** Professor Michael B Prentice, Department of Microbiology, University College Cork, Cork, Ireland

**Email:** m.prentice@ucc.ie

**Keywords:** Metabolic engineering; Synthetic biology; Bacteria; Biopolymers; Microreactors.

**Abbreviations:** BMC, bacterial microcompartment; EBPR, enhanced biological phosphorus removal ; PPK1, polyphosphate kinase; PPX, exopolyphosphatase.

**1 Abstract**

2 Processes for the biological removal of phosphate from wastewater rely on the temporary  
3 manipulation of bacterial polyphosphate levels by phased environmental stimuli. In *E. coli*  
4 polyphosphate levels are controlled via the polyphosphate-synthesizing enzyme  
5 polyphosphate kinase (PPK1) and exopolyphosphatases (PPX and GPPA), and are  
6 temporarily enhanced by PPK1 overexpression and reduced by PPX overexpression. We  
7 hypothesised that partitioning PPK1 from cytoplasmic exopolyphosphatases would  
8 increase and stabilise *E. coli* polyphosphate levels. Partitioning was achieved by co-  
9 expression of *E. coli* PPK1 fused with a microcompartment-targeting sequence and an  
10 artificial operon of *Citrobacter freundii* bacterial microcompartment genes. Encapsulation  
11 of targeted PPK1 resulted in persistent phosphate uptake and stably increased cellular  
12 polyphosphate levels throughout cell growth and into the stationary phase, while PPK1  
13 overexpression alone produced temporary polyphosphate increase and phosphate uptake.  
14 Targeted PPK1 increased polyphosphate in microcompartments 8-fold compared with non-  
15 targeted PPK1. Co-expression of PPX polyphosphatase with targeted PPK1 had little effect  
16 on elevated cellular polyphosphate levels because microcompartments retained  
17 polyphosphate. Co-expression of PPX with non-targeted PPK1 reduced cellular  
18 polyphosphate levels. Thus, subcellular compartmentalisation of a polymerising enzyme  
19 sequesters metabolic products from competing catabolism by preventing catabolic enzyme  
20 access. Specific application of this process to polyphosphate is of potential application for  
21 biological phosphate removal.

22

23

## 1 1. Introduction

2 Polyphosphate is a molecule thought to be present in all organisms [1] playing a role in  
3 cellular metabolic processes, stress response processes, virus replication and cell structure  
4 [2]. Bacterial polyphosphate accumulation underlies the enhanced biological phosphorus  
5 removal (EBPR) process, which uses microorganisms to remove inorganic phosphate (Pi)  
6 from wastewater [3]. Phosphate recovery processes are required to reduce eutrophication,  
7 the overgrowth of cyanobacteria and plants in water polluted by excess phosphorus from  
8 human activity [4], and to recycle phosphate because of the unsustainability of current  
9 natural resources beyond the next century [5]. In EBPR, cycling of wastewater sludge  
10 through aerobic and anaerobic phases of incubation lasting several hours, when continued  
11 over a period of weeks selects a bacterial consortium that has a net effect of removing  
12 phosphorus from wastewater over the cycle by accumulating it in the sludge.  
13 Polyphosphate-accumulating bacteria are key consortium components [6, 7]. Phosphate  
14 release from the consortium occurs in the anaerobic phase in parallel with consumption of  
15 volatile fatty acids, polyhydroxyalkanoate polymer formation and glycogen utilisation. In  
16 the aerobic phase stored polyhydroxyalkanoate is catabolised, glycogen replenished and  
17 phosphate taken up to form polyphosphate granules [7]. EBPR is a complex dynamic  
18 process - an uncultured bacterium *Candidatus Accumulibacter phosphatis* performs a key  
19 role in EBPR polyphosphate accumulation [8], and a functioning EBPR reactor may cease  
20 phosphate removal for unknown reasons [3, 9].

21

22 Genetic manipulation of aspects of phosphate binding, uptake and storage by a single  
23 model organism such as *E. coli* has been suggested as an alternative or additive approach

1 to biological phosphorus removal [10–13]. One issue with over-expression of a  
2 polyphosphate forming enzyme in *E. coli* has been that most of the consequent increase of  
3 polyphosphate is temporary, probably because of the existence of competing catabolic  
4 enzymes [14, 15] and phosphate release from the cells then occurs as the polyphosphate is  
5 broken down.

6

7 In *E. coli* polyphosphate kinase PPK1 (E.C. 2.7.4.1) is the enzyme responsible for  
8 assembling inorganic polyphosphate polymers in the bacterial cytoplasm by catalysing the  
9 reaction  $n\text{ATP} \rightleftharpoons (\text{polyphosphate})_n + n\text{ADP}$  [16, 17]. Although this is a reversible reaction,  
10 in *E. coli* this enzyme generally favours synthesis of polyphosphate over breakdown ( $V_{\text{max}}$   
11 ratio of 4.1) [17] (Fig. 1A). However, the balance between net accumulation and breakdown  
12 changes dynamically during culture growth and also in response to external stimuli such  
13 as [anaerobiosis](#), in part due to the action of degradative exopolyphosphatases. In this  
14 respect *E. coli* contains two such polyphosphatases that release orthophosphate from the  
15 termini of long chain polyphosphate:  $(\text{polyphosphate})_n \rightarrow (\text{polyphosphate})_{n-1} + \text{P}_i$ . The  
16 two polyphosphatases are called PPX (E.C. 3.6.1.11, sometimes referred to as PPX1) [18,  
17 19], which is encoded in the same operon as PPK1, and its homologue guanosine  
18 pentaphosphate phosphohydrolase (GPPA or PPX2) [17, 20]. GPPA (E.C. 3.6.1.40) also  
19 hydrolyses guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp) with  
20 phosphate release as part of the control of the stringent response. Both PPX and GPPA are  
21 competitively inhibited by pppGpp [21]. Consequently, amino acid starvation in *E. coli*  
22 leads to the accumulation of large amounts of polyphosphate due to the high levels of  
23 pppGpp produced as part of the stringent response [21]. In *E. coli* *ppk1* and *ppx* are adjacent

**Commented [MOU1]:** Anaerobiosis: Dictionary definition e.g. Merriam-Webster is “life in the absence of air or free oxygen”. Anaerobiosis

1 genes forming an operon and knockout of *ppx* alone has been engineered by combined  
2 knockout of *ppk1* and *ppx* with heterologous plasmid expression of *ppk1* [15, 22, 23].  
3 Elevation of polyphosphate levels in these cells rapidly declines a few hours after *ppk1*  
4 plasmid induction whether *ppx* is active or knocked out [15], showing that PPX is not the  
5 sole cause of instability in polyphosphate levels in *E. coli*. We hypothesized that an  
6 alternative approach to prevent the access of all other cytoplasmic enzymes, (not just PPX)  
7 to polyphosphate formed from recombinant PPK1 would stabilise cellular polyphosphate  
8 levels and create a phosphate-retaining phenotype. The mechanism used to achieve this is  
9 targeting of PPK1 to a recombinant bacterial microcompartment.

10

11 Bacterial microcompartments (BMCs) are proteinaceous vesicles found in certain bacteria  
12 that house specific metabolic pathways encased within a closed polyhedral shell of 100-  
13 150 nm diameter. The shells are made of thin protein sheets [24] containing pores less than  
14 1 nm in diameter [25] which can be positively or negatively charged. There are two broad  
15 groups of BMCs, those associated with the anabolic process of RuBisCO-mediated carbon  
16 fixation (carboxysomes) and those associated with catabolic fermentative processes such  
17 as 1,2-propanediol utilisation (metabolosomes) [26, 27]. Although BMCs were first seen  
18 over fifty years ago in photosynthetic cyanobacteria [28], their presence in the cytoplasm  
19 of heterotrophic bacteria was only confirmed in 1998 [29] after they were detected in thin  
20 sections of *Salmonella enterica* grown on 1,2-propanediol. In fact, around twenty per cent  
21 of bacterial genome sequences contain BMC structural genes [26], in many cases  
22 associated with enzymes of unknown function [30].

23

1 A significant proportion of bacteria therefore make a major investment in retaining and  
2 expressing large (15+ gene) operons encoding these structures and associated enzymes. It  
3 is believed the structures help mediate metabolic efficiency by selective limitation of the  
4 shell pores on the passage of substrates [31], by metabolite channelling, or other unknown  
5 mechanisms **resulting in** retention of reaction intermediates within the structure [32, 33].  
6 *Salmonella enterica* Serovar Typhimurium accrues a competitive metabolic advantage by  
7 BMC-mediated respiration of ethanolamine in a mouse colitis model [34].  
8 Enterohaemorrhagic *E. coli* obtains a similar competitive advantage from BMC-mediated  
9 ethanolamine fermentation in bovine intestinal fluid [35], favouring persistent intestinal  
10 carriage.

11

12 Recombinant BMCs using genes from *Citrobacter freundii* can be expressed  
13 heterologously in *E. coli* [36], both with and without [37] the associated interior enzymes.  
14 Peptide sequences enabling enzyme localisation to the BMC interior have been identified  
15 [37] [38]. For instance, the first 18 amino acids of PduP, P18, or the first 18 or 60 amino  
16 acids of PduD (D18 or D60) can be used as fusions to direct “foreign” proteins into the  
17 BMC [39]. Compartmentalisation of the cellular interior is a functionally transforming  
18 process often thought of as characteristic of eukaryotes [40], but specific localisation of  
19 any enzyme to a re-engineered BMC in bacteria could increase metabolic flexibility of the  
20 bacterial host enabling novel phenotypes [41]. Nanotechnological applications of  
21 biological compartment systems have included the use of viral capsids for DNA delivery  
22 [42], lumazine synthase enclosure of HIV protease [43], and the compartmentalisation of  
23 a metabolic pathway in a bacterial microcompartment [39]. We hypothesized that directing

1 PPK to a BMC would enhance polyphosphate formation within a cellular compartment and  
2 that segregation from the known degradative polyphosphatases and other cytoplasmic  
3 enzymes should stabilise accumulation of polyphosphate (Fig.1A).

4

## 5 **2. Materials and Methods**

### 6 **Strains, plasmids and culture conditions**

7 *E. coli* was grown in LB or MOPS medium [44] with either 0.5 mM or 1.0 mM K<sub>2</sub>HPO<sub>4</sub>  
8 as indicated. Expression of genes cloned into pET vectors was induced by IPTG in *E. coli*  
9 BL21 (DE3) and *E. coli* BL21 Tuner™ (DE3) (Novagen). Strains and plasmids are listed  
10 in Table 1 and oligonucleotides in Supplementary Data Table S1. **To reduce background**  
11 **expression levels of T7 RNA polymerase causing leaky gene expression prior to specific**  
12 **IPTG induction, a T7 lysozyme gene was present in all clones on pLysS or its derivative**  
13 **pSF37 (pLysS-*pduABJKNU*) unless specifically indicated.** Cultures for phosphate uptake  
14 experiments were incubated at 37 °C **and a sample growth curve is shown in**  
15 **Supplementary Data Fig. S3A.** When inducing pDuet vectors the incubation temperature  
16 was lowered to 18 °C.

17

### 18 **Molecular biology techniques**

19 Cloning strains used in this study were *E. coli* JM109 or *E. coli* TOP10 (Invitrogen). *E.*  
20 *coli* BL21(DE3) and *E. coli* BL21 Tuner™(DE3) were used for expression following  
21 standard transformation techniques [45]. For PCR reactions standard protocols were  
22 applied using an MJ Research PTC-200 Thermal Cycler for reaction cycles. Genomic DNA

1 was extracted from *E. coli* JM109 using a Wizard® Genomic DNA Purification Kit  
2 (Promega). Plasmid constructs were sequenced commercially (GATC Biotech).

3

#### 4 **Targeting of PPK1 to microcompartments**

5 The cloning strategy used to engineer plasmids for targeting of PPK1 to the  
6 microcompartment is summarised in Supplementary Data Fig.S1. The *ppk1* gene coding  
7 for polyphosphate kinase (PPK1) was PCR-amplified with a proofreading DNA  
8 polymerase (Bioline High Velocity Polymerase, Bioline UK, London), using genomic  
9 DNA from *E. coli* JM109 as template, using the forward primer PPK1-F and the reverse  
10 primer PPK1-R (Table S1). The PCR product was digested with *SacI* and *HindIII*  
11 (Fermentas) followed by ligation into pET23b-*pduP18-gfp* digested with *SacI* and *HindIII*.  
12 The gene encoding the GFP was replaced by *ppk* with retention of the *pdu* localization  
13 sequence *p18*. The ligation product was transformed into *E. coli* Top 10 electrocompetent  
14 cells (Invitrogen) by electroporation. The new vector, named pML001 (pET23b-*pduP18*-  
15 *ppk1*), was extracted and the *ppk1* insert was sequenced (GATC-Biotech) to confirm no  
16 mutation had occurred. Two constructs, pML001 and pLysSpduABJKNU (pSF37),  
17 expressing empty *pdu* BMCs [37]), were co-transformed into *E. coli* BL21 (DE3) by heat  
18 shock.

#### 19 **Co-expression of targeted and untargeted PPK1 and PPX**

20 The pCOLADuet-1 coexpression vector (Novagen) system encoding two multiple cloning  
21 sites (MCS) each preceded by a T7 promoter, *lac* operator, and ribosome binding site was  
22 used to express targeted and untargeted *E. coli* PPK1 and PPX (*ppx* amplified from *E. coli*

1 JM109) in combination (pYY005, pYY007, pYY008) and alone (pYY002, pYY010) (see  
2 Table 1).

### 3 **Microcompartment purification**

4 Recombinant BMCs were extracted from *E. coli* by modification of the method of Sinha  
5 et al [46]. A single colony of *E. coli* BL21(DE3) containing the plasmid encoding  
6 microcompartment proteins (pLysS-*pduABJKNU*) was picked and grown in 200 ml of LB  
7 to an OD<sub>600</sub> of 0.4 followed by protein induction with 0.4 mM IPTG. Cells were harvested  
8 at OD<sub>600</sub> 1.0-1.2 and washed twice with 40 ml of buffer A (50 mM Tris-HCl (pH 8.0), 500  
9 mM KCl, 12.5 mM MgCl<sub>2</sub>, 1.5% 1,2-PD). Cells (1 g wet weight) were resuspended in a  
10 mixture of 10 ml of buffer A and 15 ml of B-PER II bacterial protein extraction reagent  
11 supplemented with 5mM mercaptoethanol, Complete Protease Inhibitor Cocktail (Roche)  
12 at the manufacturer's recommended working dilution, 25 mg of lysozyme, and 2 mg of  
13 DNase I. The suspension was incubated for 30 min on a shaking platform at room  
14 temperature and on ice for 5 min. After initial removal of cell debris by centrifugation at  
15 12,000 x *g* for 5 min at 4°C (repeated twice), the BMC fraction was pelleted by spinning  
16 at 20,000 x *g* for 20 min at 4°C. The pellet was washed once with a mixture of 4 ml of  
17 buffer A and 6 ml of B-PER II and resuspended in 0.5 ml of buffer B (50 mM Tris-HCl pH  
18 8.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1% 1,2-PD) containing protease inhibitor. Remaining cell  
19 debris was removed by centrifugation for 1 min at 12,000 x *g* at 4°C (repeated three times).  
20 Aliquots (50 µg) of extracted protein were separated by SDS-PAGE using a 15%  
21 polyacrylamide gel under denaturing conditions in a MiniProtean apparatus (Bio-Rad) and  
22 stained with Coomassie Brilliant Blue R250 (Fig.1B). To confirm the identity of proteins  
23 peptide fingerprinting by MALDI-TOF-MS (matrix assisted laser desorption/ionization-

1 **time of flight mass spectroscopy**) was carried out as previously described [47].  
2 Microcompartments used in ATP regeneration assays (Fig. 1C,D) and whole cell  
3 polyphosphate assays (Fig 2) were initially extracted with CelLytic B (Sigma-Aldrich)  
4 instead of B-PER II because of reported efficacy of this reagent for polyphosphate  
5 extraction [48]. Later comparisons of B-PER II extractions and CelLytic B extractions had  
6 shown little difference in measured polyphosphate levels and B-PER II was used for  
7 microcompartment extractions shown in Fig. 3. Micrococcal nuclease 2 mg (Sigma-  
8 Aldrich) prepared with calcium buffer was substituted for DNase 1 for all  
9 microcompartment extractions where polyphosphate was assayed because of the potential  
10 adverse effect of Mg<sup>2+</sup> containing buffers on polyphosphate [49].

#### 11 **ATP regeneration assay to detect PPK1 enzyme activity in microcompartments**

12 A combination of two previously described PPK1 assay methods [48, 50] using luciferase  
13 to detect ATP produced from polyphosphate by PPK1 was used as a biochemical screen  
14 for the presence of PPK1 and polyphosphate in microcompartment fractions. Briefly, to  
15 assay relative PPK1 content 20 µL of BMC extract was added to a 100 µL reaction mixture  
16 containing: ultrapure ADP (ATP-free, Cell Technology Inc, Ca), 30 mM MgCl<sub>2</sub>, 1% (w/v)  
17 Polyphosphate (Sigma), 50 mM Tris-HCl (pH 7.8). The reaction mixture was diluted 1:100  
18 in 100 mM Tris-HCl pH 8.0, 4 mM EDTA, of which 0.1 mL was added to 0.1 mL of  
19 luciferase reaction mixture from ATP Bioluminescence Assay Kit CLS II (Roche).  
20 Luminescence was measured by using a luminometer (Luminoskan, Thermo Labsystems).  
21 A standard curve for ATP by dilution in 100 mM Tris-HCl pH 8.0 containing 4 mM EDTA  
22 was used to determine PPK1 activity of the extracts (Fig. 1C). To assay relative

1 polyphosphate content, the same reaction omitting added polyphosphate was performed  
2 (Figure 1D).

### 3 **Determination of polyphosphate content of whole cells and extracted** 4 **microcompartments**

5 Polyphosphate concentrations in whole cells presented in Fig. 2A were determined  
6 following lysis of pelleted cells from 10 ml of cultures. A metachromatic assay was  
7 employed using the 530/630nm absorbance ratio of 10  $\mu$ L of lysate added to 1 mL of  
8 toluidine dye solution (6 mg/L toluidine blue in 40 mM acetic acid) as described [51]. In  
9 later experiments (Fig. 3) polyphosphate was determined by a higher-yielding method  
10 using 4'-6-diamidino-2-phenylindole (DAPI) as described previously [52]. For this method  
11 cells were harvested by centrifuging at 5000 x g for 10 min at 4° C. After washing in 50  
12 mM HEPES buffer (pH 7.5) the cell pellet or purified microcompartment sample was  
13 frozen at -20° C followed by defrosting at room temperature. Cell pellets/purified  
14 microcompartments were resuspended in HEPES buffer at an appropriate dilution to ensure  
15 that the cellular polyP concentration was in the linear range of the DAPI assay (0-6 g  
16 polyP/ml). Total assay volume was 300 $\mu$ l which included 100  $\mu$ L of polyP containing  
17 samples and 200  $\mu$ L of DAPI assay buffer containing 150 mM KCl, 20 mM HEPES-KOH  
18 (pH 7.0) and 10  $\mu$ M DAPI solution. After a 10 min incubation at room temperature DAPI  
19 fluorescence was measured with a plate reader equipped with excitation and emission  
20 filters of 420 nm and 550 nm respectively. **This method was also used to assess the efficacy**  
21 **of heat treatment in releasing polyphosphate from cells (Supplementary Data Fig. S4).**

22

1 A polyphosphate standard curve was prepared using sodium phosphate glass Type 45  
2 (S4379 Aldrich) and sodium hexametaphosphate (SX0583). Protein concentration of cell  
3 extracts was measured using a 10 $\mu$ L sample by Bradford assay [53], with Coomassie Plus  
4 Protein Assay Reagent (Pierce) with bovine serum albumin as the standard resuspended in  
5 the same buffer as the sample.

6

7 **Phosphate uptake** by bacteria from defined media was determined as follows (Fig. 2B).

8 Strains were cultured in LB to OD<sub>600</sub> 0.4-0.6 and then induced for 1 hr with 0.5 mM of  
9 IPTG before being transferred to MOPS medium pH 5.5 [44] containing 0.01 mM iron and  
10 0.5 mM potassium phosphate, at an OD<sub>600</sub> of 0.2. Incubation at 37°C was continued up to  
11 48 hrs with intermittent sampling of 0.2 mL. Samples were pelleted by centrifugation and  
12 the MOPS medium was analysed for orthophosphate concentration. The cell pellet was  
13 analysed for polyphosphate content and protein concentration as described above.  
14 Orthophosphate was assayed using a molybdovanadate colorimetric method [54]. 0.2 mL  
15 of molybdovanadate solution (Reagecon, cat no: 1056700) was added to 5 mL of culture  
16 medium, mixed and incubated at room temperature for 5 min. Optical density of 1 mL was  
17 measured at 430 nm against a blank of 4% molybdovanadate in distilled water and a  
18 calibration curve of potassium phosphate in MOPS.

19

#### 20 **Light Microscopy**

21 Polyphosphate granules were visualised (Fig. 4) by Neisser's stain using Chrysoidin  
22 counterstain [55].

23

1 **Electron microscopy for parallel electron energy loss spectroscopy (PEELS) and**  
2 **element mapping by electron spectroscopic imaging (ESI)**

3 Unstained cells were fixed in 3% (v/v) glutaraldehyde, 10 mM Hepes, pH 7.3 (Sigma),  
4 dehydrated in an acetone-series and embedded in epoxy resin (Spurr, hard mixture; [56]),  
5 as described [57]. For elemental analysis 30 - 40 nm ultrathin sections (otherwise 90 nm  
6 for general ultrastructure) were sectioned with a Reichelt-Jung ultramicrotome (Leica,  
7 Vienna, Austria), equipped with a diamond knife and were picked up with 300 mesh Cu-  
8 grids. Electron micrographs were recorded in the elastic brightfield mode (slit width: 10  
9 eV) with an EF-TEM (operated in general at 120 kV acceleration voltage), equipped with  
10 an in-column Omega-type energy filter (LIBRA120 plus, Zeiss, Oberkochen Germany), in  
11 a magnification range from x 4000 to x 32000 with a bottom-mount cooled 2048 x 2048  
12 CCD camera (SharpEye; Tröndle, Moorenweis, Germany).

13

14 **Parallel electron energy loss spectroscopy (PEELS)**

15 Spot-PEELS were recorded within electron dense cytoplasmic inclusion bodies. Spot-size  
16 was set to 16 nm and the objective aperture was 60  $\mu\text{m}$  (spectrum magnification: x100;  
17 energy range: 67 – 290 eV; recording time: 10 s ; emission current: 1  $\mu\text{A}$ ) and the spectrum  
18 energy resolution was about 1.6 eV at zero-loss (FWHM). Recorded PEELS data were  
19 corrected for background, applying the ‘potence’ underground function of the EsiVision  
20 Pro Software (EsiVision Pro, Vers. 3.2; SIS – Soft Imaging Systems, Munster, Germany)  
21 and were ‘medium’-filtered (settings: 1.5 eV width).

22

23 **Element mapping by electron spectroscopic imaging (ESI)**

1 Phosphorus mapping was performed as previously described [57] with unstained 35 nm  
2 ultrathin sections. According to the '3-window method' energy-windows were set to a  
3 dedicated energy loss for the P-L23 edge, as it was given by the corresponding first  
4 intensity maximum from the spot-PEELS, i.e. 138 eV (W1: 125 eV; W2: 115 eV). The  
5 energy selective slit was set to 6 eV width, and images were recorded with an illumination  
6 aperture of 0.63 mrad, an emission current of 1  $\mu$ A, a 60  $\mu$ m objective aperture, and a  
7 nominal magnification of x 6300. Background subtraction for calculating the phosphorus  
8 element map was performed by the 'multiwindow exponential difference' method.

9

### 10 **3. Results**

#### 11 **PPK1 is targeted to recombinant BMCs**

12 The localisation of PPK1 to a recombinant BMC was achieved by engineering a fusion  
13 between the known targeting peptide P18 and the N-terminus of PPK1, resulting in P18-  
14 PPK1. Recombinant BMCs with the associated P18-PPK1 were isolated from a strain co-  
15 producing the *C. freundii* Pdu shell proteins PduABJKNU (pSF37) and P18-PPK1  
16 (pML001). Analysis of the purified BMC fraction by SDS-PAGE revealed the presence of  
17 P18-PPK1 together with the BMC-associated shell proteins (Fig. 1B).

18

19 A functional assay designed to maximise PPK1's ATP breakdown function was employed  
20 to determine the activity of PPK1 when it was directed to the BMC. In comparison to  
21 BMCs isolated from cells producing only empty BMCs, or empty BMCs and non-targeted  
22 PPK, the purified BMCs from the strain co-producing BMCs and P18-PPK1 generated  
23 over twenty-fold more ATP per mg of protein from added polyphosphate (Fig.1C). There

1 was little activity in the equivalent protein fraction prepared using the same  
2 microcompartment purification protocol from cells producing only P18-PPK1 in the  
3 absence of BMCs. This showed that polyphosphate kinase activity had been transferred to  
4 the microcompartments by enzyme targeting.

5

6 The same ATP regeneration assay was repeated in the absence of any added exogenous  
7 polyphosphate (Fig.1D). Any ATP generated in this assay would therefore reflect the  
8 amount of endogenous polyphosphate within the fraction. The BMC fraction from the cells  
9 that co-produced both the BMCs and P18-PPK1 generated more than twice as much ATP  
10 as control BMC fractions from cells expressing empty BMCs or BMCs with non-targeted  
11 PPK1 (Fig. 1D). This result indicates that the BMCs from strains co-expressing targeted  
12 PPK1 had increased levels of polyphosphate, compatible with localisation of PPK1 to the  
13 microcompartment and formation of polyphosphate in situ.

14

#### 15 **Effect of PPK1 targeting effect on polyphosphate content and phosphate uptake,**

16 Targeted PPK1 with co-expressed BMCs conferred a distinct cellular phenotype. DAPI  
17 negative staining of polyphosphate extracted from whole cells cultured at 37 °C, size-  
18 separated on a PAGE gel (Supplementary Data Figure S2), showed that the polyphosphate  
19 detected in strains over-expressing either P18-PPK1 alone or P18-PPK1 and BMCs  
20 exceeded the length of the sodium phosphate glass Type 45 polyphosphate control. This  
21 indicates that long chain polyphosphate is present in these strains. No qualitative difference  
22 in chain length was detected between these two clones but long chain polyphosphate in the  
23 *E. coli* strain over expressing P18-PPK1 and recombinant BMCs was less evident at time

1 zero but persisted to a later phase of growth (Fig. S2) than in cells expressing P18-PPK1  
2 alone. The cells expressing P18-PPK1 alone contained long chain polyphosphate before  
3 IPTG induction, probably because of promoter leakage (Supplementary Data Figure S2).  
4 No long chain polyphosphate was detected in the *E. coli* control.

5

6 A simultaneous quantitative assay of the cellular polyphosphate and phosphate content of  
7 the culture supernatant from the cultures used in the polyphosphate chain length assay was  
8 also undertaken. Here, increased phosphate uptake from culture medium was observed in  
9 comparison to the host *E. coli* control (Fig. 2B) by both the P18-PPK1-expressing strain  
10 and the strain expressing both P18-PPK1 and BMCs. A maximal uptake of approximately  
11 0.25 mM at 20 hours was observed for both strains. However, the P18-PPK1-expressing  
12 strain returned a third of this phosphate to the supernatant after 48 hours, while the strain  
13 expressing both P18-PPK1 and BMCs returned less than 9% of phosphate taken up by 48  
14 hours. Correspondingly, the cell-associated polyphosphate levels of the P18-PPK1 strain  
15 were maximal at 20 hours and declined thereafter (Fig. 2A), while the P18-PPK1 and  
16 BMC-expressing strain retained approximately the same level of cell associated  
17 polyphosphate at 48 hours as at 20 hours. The polyphosphate time course was repeated  
18 with a simultaneous growth curve and both co-expression of targeted PPK1 and  
19 recombinant microcompartments in *E. coli* expression of targeted PPK1 alone caused a  
20 similar mild growth retardation to (Supplementary Data Fig. S3).

21

22 **BMCs protect endogenous polyphosphate from exogenous polyphosphatases**

1 Simultaneous expression of polyphosphate-forming PPK1 and the exopolyphosphatase  
2 PPX from the pDuet vector in BMC-expressing *E.coli* strains was used to examine the  
3 effect of BMC-targeting of these enzymes on cellular polyphosphate accumulation. The  
4 induction of non-targeted PPK1 from the pDuet vector increased whole cell polyphosphate  
5 levels 5-fold in comparison to control cells containing the BMC shell protein operon and  
6 the pDuet vector with no enzyme insert (the enzyme-free control, Fig. 3). It did not increase  
7 the polyphosphate content of co-expressed recombinant microcompartments when  
8 compared to the enzyme-free control. However, P18-PPK1, when co-produced with the  
9 BMCs, increased polyphosphate levels in the BMC fraction 8-fold in comparison to the  
10 enzyme-free control, while giving a similar overall 5-fold increase in whole cell  
11 polyphosphate to that seen with expression of non-targeted PPK1.

12

13 Co-expression of non-targeted polyphosphatase PPX with non-targeted PPK1 reduced  
14 whole cell polyphosphate levels by 50% compared with non-targeted PPK1 expression  
15 alone, with little effect on polyphosphate levels in the microcompartment fraction. Co-  
16 expression of non-targeted PPX and BMC-targeted P18-PPK1 reduced whole cell  
17 polyphosphate levels by 22% and BMC-associated polyphosphate by 18% when compared  
18 with microcompartment targeted PPK1 alone. BMC-associated polyphosphate was still at  
19 least 2.5 times greater than in cells co-expressing non-targeted PPK1 in the presence or  
20 absence of non-targeted PPX. Co-expression of BMC targeted P18-PPK1 with PPX  
21 targeted to the microcompartment using a different tag (D60) reduced the BMC-associated  
22 polyphosphate content by 50% in comparison to the BMC-targeted P18-PPK1 alone, while  
23 reducing whole cell polyphosphate by 22%. **The D60 tag was used empirically, to avoid**

1 **risking potential interference with targeting caused by using the same tag for two enzymes,**  
2 **subsequent experiments outside the scope of this manuscript showed differential tagging**  
3 **was unnecessary.** These data suggest that the BMC-targeting of PPK1 results in the  
4 synthesis of polyphosphate that is located primarily within the BMC fraction of the cell  
5 and is relatively inaccessible to cytoplasmic co-expressed PPX, but more accessible to  
6 BMC-targeted PPX. **However, this polyphosphate is released from cells by heat treatment**  
7 **(Supplementary Data Figure S4).**

8

### 9 **Microscopy**

10 Blue-black granules were apparent with Neisser's stain in a proportion of all cells  
11 overexpressing P18-PPK1, but not the *E. coli* BL21 (DE3) insert-free control or without  
12 any targeted enzyme (Fig. 4). These appearances are consistent with the accumulation of  
13 intracellular polyphosphate in *E. coli* cells with increased PPK1 activity. All cells  
14 overexpressing P18-PPK1 showed a heterogeneous granule phenotype, with a proportion  
15 of non-toluidine blue staining cells in all fields.

16

17 *E. coli* expressing the recombinant microcompartment and P18-PPK1 retained the  
18 polyphosphate staining at 44 hours whereas cells expressing P18-PPK1 without the  
19 recombinant microcompartment showed reduced staining after 40 hours (Fig. 4).

20 All *E. coli* expressing the recombinant microcompartment had a proportion of cells which  
21 were greatly elongated. All *E. coli* forming multiple polyphosphate granules tended to be  
22 larger than the non-granulated cells, presumably because of distension by the granules.

1 However, the largest cells were seen with **co-expression** of recombinant  
2 microcompartments and **the tagged P18-PPK1 enzyme**.

3

#### 4 **Electron-loss spectroscopic analysis by Energy-filtered Transmission Electron** 5 **Microscopy (EFTEM).**

6

7 Increased phosphorus deposition was detected in all cells expressing recombinant *E.coli*  
8 PPK1 by EFTEM (Fig. 5C,D,E,F), verified from PEELS measurement (see below),  
9 compared with control *E. coli* strains with no recombinant gene expression (Fig. 5A) or  
10 expressing microcompartment genes (Fig. 5B). In cells expressing PPK1 alone, most  
11 phosphate signal was represented by particles <5 nm, but some large homogeneous masses  
12 > 200 nm with plane edges were visible (Fig. 5C) in a few cells. In cells expressing targeted  
13 PPK1 and a recombinant microcompartment operon, in addition to signals from particles  
14 <5 nm, multiple phosphate signals from particles 50-100 nm were present (Fig.5D,E,F)  
15 and in some cases large circular masses/crescents > 300 nm were present (Fig. 5D,F). These  
16 large masses were not homogeneous and appeared composed of small particles and the  
17 cells containing them were enlarged. These images appeared similar to light microscopy  
18 observations (Fig. 4D,H,L).

19

#### 20 **Parallel electron energy loss spectroscopy (PEELS)**

21 Spot-PEELS recorded from dark inclusions apparent as electron dense regions about 100  
22 nm in diameter (Fig 5G), confirmed they contained phosphate, verified from the  
23 characteristic ELNES-fingerprint (Energy-Loss Near-Edge Structure) of reference spectra

1 that were recorded from sodium polyphosphate (Fig.5G). The largest polyphosphate  
2 inclusion in figure 5E, shown in yellow, is magnified in the inset of the spot-PEELS (Fig.  
3 5G); here the 16 nm beam spot and its position are indicated (white circle).

#### 1 4. Discussion

2 Polyphosphate accumulation is the basis of the enhanced biological phosphorus removal  
3 (EBPR) process, which uses microorganisms to remove inorganic phosphate (Pi) from  
4 wastewater. Accumulation occurs in aerobic conditions as intracellular polyphosphate [8,  
5 12] is released as Pi in anaerobic conditions [58] or when the consortium is supplied with  
6 organic carbon or heated [12]. The best characterized enzyme responsible for  
7 polyphosphate synthesis (PPK1), originally found in *E. coli* [59], can only be detected in  
8 silico in the genome sequences of a minority of bacterial genera [60]. The enzyme  
9 responsible for polyphosphate synthesis in most bacteria therefore remains to be identified  
10 [60].

11

12 In *E. coli*, polyphosphate accumulation in wild-type strains occurs with amino acid  
13 starvation or in the stationary phase [21, 50, 61]. Large amounts of polyphosphate  
14 accumulate only if the copy number of *ppk1* is increased [14], or a heterologous *ppk* gene  
15 is supplied [62], or the phosphate regulatory gene *phoU* is mutated [63, 64]. Even in *E. coli*  
16 strains overexpressing *ppk1*, initial accumulation of polyphosphate is known to be partially  
17 or completely reversed as the cells reach stationary phase [14, 15]. Because this also occurs  
18 in *E. coli* overexpressing *ppk1* with no chromosomal functioning *ppx* gene it has been  
19 suggested to be due to either product-induced reversal of the PPK-catalysed reaction, or  
20 the activity of another phosphatase enzyme present in the cytoplasm [15]. We observed a  
21 similar reversal of polyphosphate accumulation in our overexpressing *ppk1* clone,  
22 accompanied by increasing Pi in the culture supernatant (Fig. 2). This did not occur when

1 the *ppk1* gene was engineered to encode an N-terminal BMC localisation sequence (*p18-*  
2 *ppk1*) and was expressed in trans with an operon encoding an empty BMC.

3

4 Cells co-producing P18-PPK1 and the empty BMC had a different phosphorus distribution  
5 by EFTEM (Fig 5D,E,F) to those expressing PPK1 alone (Fig. 5C), containing single or  
6 agglomerated particles in the BMC size range. BMC extractions show the presence of  
7 metabolically active PPK1 (Fig.1C,D) and polyphosphate in the BMC fraction  
8 (Fig.1D, Fig.3) when PPK1 is microcompartment-targeted in this way. Our results suggest  
9 that targeting of PPK1 to a bacterial microcompartment still allows access of the small  
10 molecule substrate ATP to the enzyme (Figure 1A), but effectively stabilises the large  
11 polymer polyphosphate product (Fig. 2B).

12

13 We hypothesized that this stabilisation results from reduced access of PPX, GPPA or  
14 other cytoplasmic phosphatases to the polyphosphate produced by BMC-targeted PPK1.

15 To confirm this we carried out co-expression experiments of PPK1 with PPX (Fig. 3).

16 Co-production of PPX with PPK1 resulted in lower cellular polyphosphate levels than  
17 expression of *ppk1* alone (Fig. 3), as has been previously reported [65]. This reduction in  
18 total cellular polyphosphate was partially prevented by BMC-association of PPK1, due to  
19 increased levels of polyphosphate in the BMC fraction. BMC-targeting of PPK1 therefore  
20 results in the synthesis of polyphosphate that is located primarily in the BMC fraction of  
21 the cell. Polyphosphate in the BMC fraction is inaccessible to cytoplasmic co-expressed  
22 PPX. Adding BMC targeting to PPX using an alternative tag (D60-PPX) co-expressed  
23 with targeted PPK1 (P18-PPK1) partially reverses the increase in polyphosphate levels in

1 the BMC fraction conferred by targeted PPK1, presumably by increasing access of the  
2 PPX to polyphosphate in the BMC fraction. This suggests that the mechanism of  
3 stabilisation of polyphosphate conferred by BMC targeting of PPK1 involves reduced  
4 access by cytoplasmic phosphatases.

5

6 Other examples of such macromolecular association of enzymes exist. A variant of  
7 lumazine synthase was recently employed to encapsidate HIV protease within an *E. coli*  
8 host [43] facilitating recombinant synthesis of this potentially toxic enzyme by separating  
9 it from the remaining cytoplasm. Lumazine synthase compartments are genetically  
10 unrelated to BMCs involved in catabolic metabolism, and form pentameric components  
11 form smaller 30-40 nm icosahedral structures that more closely resemble viral capsids [66].  
12 The enzyme is bound to part of the shell molecule forming the inner surface by an  
13 electrostatic mechanism [43, 67], (N-terminal fusion displays it on the outside [68]).  
14 Enzymically active inclusion bodies can be formed within bacterial cells by C-terminal  
15 attachment of short self-assembling peptide sequences [69], or N-terminal fusion with a  
16 self aggregating protein [70] but these enzymes are not enclosed within a structure  
17 accessed via pores. Subcellular localisation of enzymes catalysing successive reactions in  
18 a metabolic pathway to peroxisomes in fungi [71] or BMCs [39] can promote product  
19 formation.

20

21 Our results demonstrate that P18-PPK1 is targeted to a recombinant BMC. The  
22 observation that polyphosphate accumulates within the BMC suggests that targeted PPK1  
23 is internalised within the structure and remains functional, generating polymeric product.

1 ATP must be able to enter the recombinant BMC to allow it to act as one of the substrates  
2 for the P18-PPK1 enzyme (Fig. 1A). However, this is not surprising as the native Pdu  
3 BMC must allow ATP access as it is required by PduO (located within the  
4 microcompartment) for the regeneration of the coenzyme form of cobalamin needed by  
5 the diol dehydratase complex [72]. The association of PPK1 with the BMC however  
6 leads to sequestration of the enzyme's metabolic product, presumably because its size  
7 does not allow it to leave the BMC by the same route by which the enzyme substrate  
8 ATP arrived. Protection of the polyphosphate product from catabolism from cytosolic  
9 enzymes is therefore achieved, illustrating a general mechanism by which BMC can be  
10 used to re-engineer cellular metabolism. The specific polymer generated, polyphosphate,  
11 is an important intermediary in the enhanced biological phosphate removal (EBPR)  
12 process employing environmental bacteria to remove phosphate from wastewater [3, 12]  
13 and has industrial applications [73]. We have shown heat treatment releases  
14 microcompartment-located polyphosphate from bacteria (Data Supplement FigS4) as it  
15 does from standard EBPR sludge bacteria [74], so recovery of sequestered  
16 polyphosphate is readily achievable. EBPR requires prolonged cycles of aerobic and  
17 aerobic incubation to operate. The ability to stabilise polyphosphate produced in a single  
18 growth phase so that phosphate is not returned to the cell exterior could lead to a  
19 streamlined process with a single phase of incubation. This would require transfer of the  
20 recombinant microcompartment and targeted polyphosphate kinase from *E. coli* to a more  
21 environmentally robust organism, but horizontal transfer of both these components is  
22 straightforward. Further evaluation of the properties of any recombinant organism of this

1 type in a closed system would be required before any assessment of the safety of  
2 environmental release.

3

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18 US Patent US9187766 (B2) (also currently WO2013045562 (A1), EP2760883 (A1))  
19 “Accumulation of metabolic products in bacterial microcompartments”.

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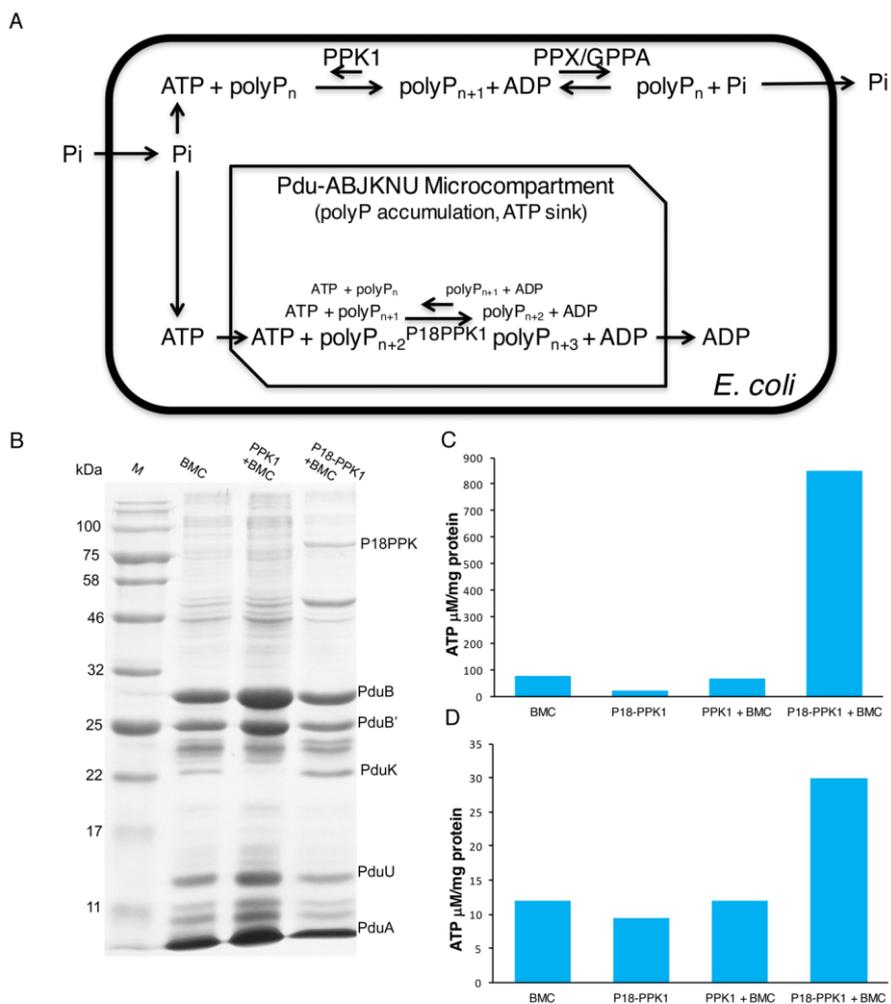
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**Table 1**  
**Plasmids and strains used in this study**

Plasmids and strains	Genotype*	Source
<b>Plasmids</b>		
pET23b	pBR322, T7 Ap	Novagen
pCOLADuet-1™	ColA ori lacI T7lac Kan <sup>r</sup>	Novagen
pLysS	p15A Cam <sup>R</sup>	Novagen
pET23b- PduP18-GFP	pET23b with <i>pduP18</i> <sup>†</sup> leader sequence and <i>gfp</i> <sup>▲</sup>	Prof. Martin Warren, University of Kent
pLysS-PduABJKNU (pSF37)	Cam <sup>R</sup> , Tet <sup>R</sup> <i>pduABJKNU</i> <sup>†</sup>	[37]
pML001	pET23b with <i>pduP18</i> <sup>†</sup> - <i>ppk1</i> fusion without <i>gfp</i> <sup>▲</sup>	This study
pML002	pET23b- <i>ppk1</i>	This study
pCOLADuetPPK (pYY002)	pCOLADuet-1 with <i>ppk1</i>	This study
pCOLADuetP18PPK (pYY010)	pCOLADuet-1 with <i>pduP18</i> <sup>†</sup> - <i>ppk1</i> fusion	This study
pCOLADuetPPXPPK (pYY005)	pCOLADuet-1 with <i>ppk1</i> and <i>ppx</i>	This study
pCOLADuetP18PPKPPX (pYY007)	pCOLADuet-1 with <i>pduP18</i> <sup>†</sup> - <i>ppk1</i> fusion and <i>ppx</i>	This study
pCOLADuetD60PPXP18PPK (pYY008)	pCOLADuet-1 with <i>ppk1</i> and <i>pduD60</i> <sup>†</sup> - <i>ppx</i> fusion	This study
<b>Strains</b>		
<i>E. coli</i> JM109	<i>endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB</i> <sup>+</sup> $\Delta$ ( <i>lac-proAB</i> ) <i>e14- hsdR17</i> ( <i>rK</i> <sup>+</sup> <i>mK</i> <sup>+</sup> )	Promega
<i>E. coli</i> Top 10	<i>F- mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZAM15</i> $\Delta$ <i>lacX74 nupG recA1 araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galE15 galK16 rpsL</i> ( <i>Str</i> <sup>R</sup> ) <i>endA1</i> $\lambda$ <sup>-</sup>	Invitrogen
<i>E. coli</i> BL21 (DE3)	<i>F</i> <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( <i>r<sub>B</sub> m<sub>B</sub></i> ) <i>gal dcm</i> (DE3)	Stratagene
<i>E. coli</i> Tuner (DE3)	<i>F</i> <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( <i>r<sub>B</sub> m<sub>B</sub></i> ) <i>gal dcm lacY1</i> (DE3)	Stratagene

\*All inserts from *E. coli* JM109 unless specified <sup>†</sup>From *Citrobacter freundii* <sup>▲</sup>From *Aequorea victoria*

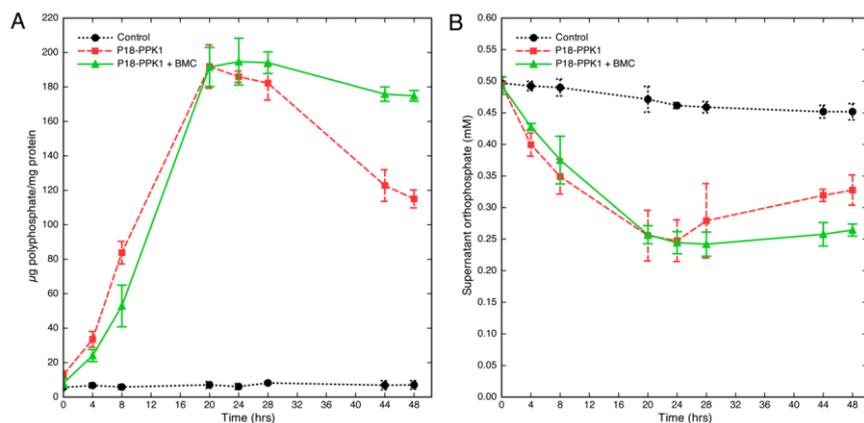
Figures



**Fig. 1. The effect of microcompartment-targeting of polyphosphate kinase (PPK1) on polyphosphate metabolism in *E. coli***

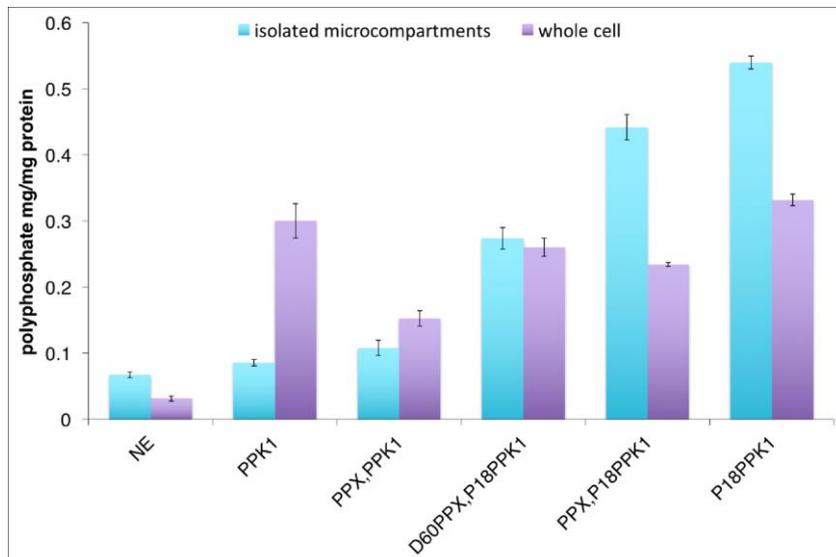
A. Proposed mechanism of increasing polyphosphate content of *E. coli* by microcompartment-targeting of polyphosphate kinase (PPK1). B. SDS-PAGE gel of microcompartment samples isolated from various *E. coli* BL21 (DE3) strains. M: MW marker. BMC: empty microcompartments only, (pSF37). PPK1 + BMC : non-targeted PPK1 plus microcompartments (pML002 + pSF37). P18-PPK1+ BMC: microcompartment-targeted P18-PPK1 plus microcompartments (pML001+ pSF37). C. Polyphosphate kinase assay on isolated microcompartments with addition of ADP and polyphosphate. D. Polyphosphate kinase assay on isolated microcompartments with addition of ADP alone. In C and D the

second column represents a control microcompartment extraction procedure from **pLysS containing** cells expressing P18-PPK alone without microcompartments.



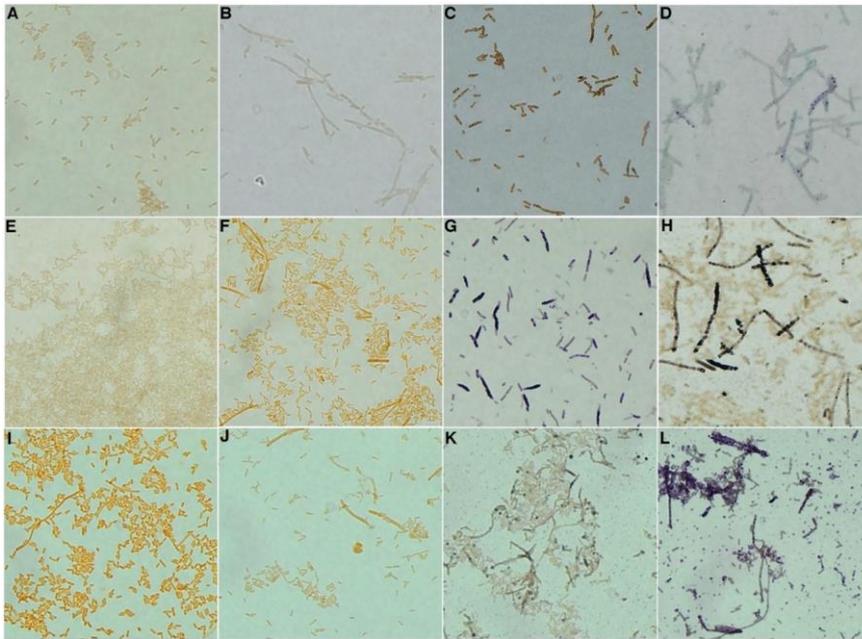
**Fig. 2. Co-expression of targeted PPK1 and recombinant microcompartments in *E. coli* results in stable orthophosphate uptake and polyphosphate retention.**

**A. Polyphosphate content of whole cells over 48 hours. B. Orthophosphate concentration in culture medium over 48 hours.** Dashed line with filled circles: *E. coli* BL21 (DE3) pLysS control. Continuous red line with filled squares : P18-PPK1, microcompartment-targeted PPK1 (pML001 + pLysS). Continuous green line with filled triangles : P18-PPK1 + BMC, microcompartment-targeted P18-PPK1 plus microcompartments (pML001 + pSF37). Error bars represent standard deviation of three independent observations.

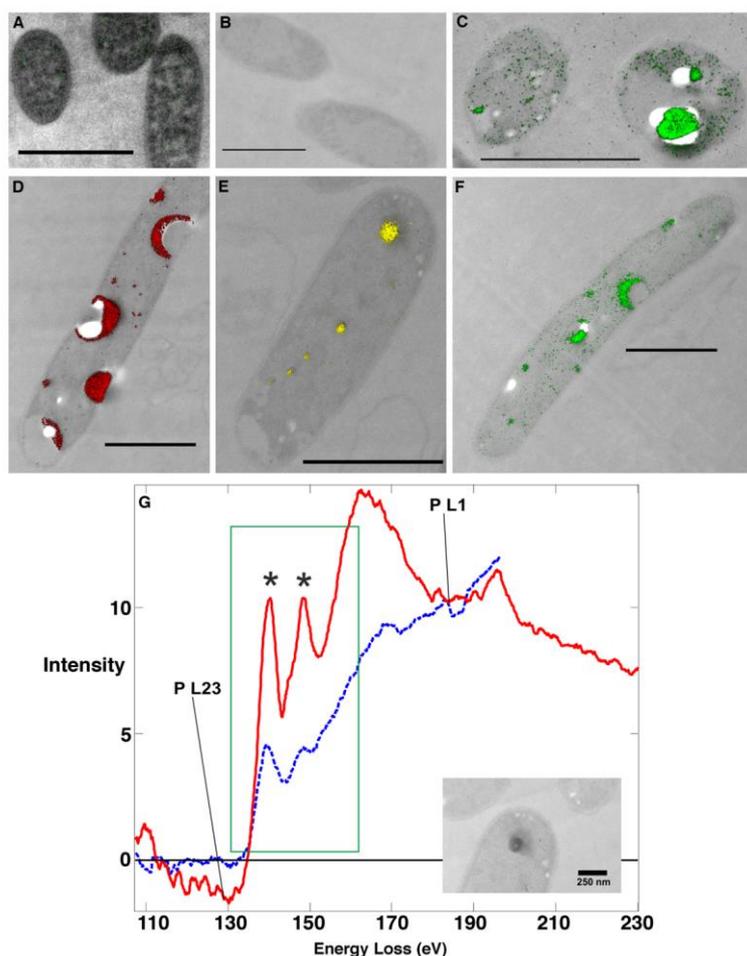


**Fig. 3. Co-expression of microcompartment-targeted PPK1 and recombinant microcompartments in *E. coli* increases the polyphosphate content of isolated microcompartments and protects polyphosphate from co-expressed cytoplasmic polyphosphatase.**

Polyphosphate content of isolated microcompartments (blue bars) and whole cells (purple bars) measured with a DAPI assay following overnight culture. All *E. coli* BL21 (DE3) strains were expressing microcompartments (pSF37) and in addition various combinations of targeted and un-targeted PPK1 and PPX. BMC: microcompartments only, (pSF37). PPK1 + BMC: non-targeted PPK1 and microcompartments, (pML002 + pSF37). PPK1 + PPX + BMC: non-targeted PPK1 and non-targeted PPX and microcompartments (pYY005 + pSF37). P18-PPK1 + D60PPX + BMC : targeted PPK1 and targeted PPX and microcompartments (pYY08+ pSF37) . P18-PPK1 + PPX + BMC: targeted PPK1 and non-targeted PPX and microcompartments (pYY07 + pSF37). P18-PPK1 + BMC: targeted PPK1 and microcompartments (pYY010 + pSF37). Error bars represent standard deviation of three independent observations.



**Fig. 4. Co-expression of targeted PPK1 and recombinant microcompartments in *E. coli* results in cytoplasmic polyphosphate granule formation persisting into stationary phase.** Light microscopy of Neisser stained fixed cells (toluidine blue and chrysoidine counterstain, polyphosphate appears purple-black, predominantly yellow cells are polyphosphate-free). A,E,I: control *E. coli* BL21 (DE3) pLysS. B,F,J: *E. coli* BL21 (DE3) BMC, microcompartments only (pSF37). C,G,K: *E. coli* BL21 (DE3) P18-PPK1, targeted PPK1 (pML001 + pLysS). D,H,L: *E. coli* BL21 (DE3) P18-PPK1 + BMC, targeted PPK1 plus microcompartments (pML001 + pSF37). Incubation time in MOPS : A,B,C,D 4 hours, E,F,G,H 18 hours, I,J,K,L 44 hours.



**Fig. 5. Phosphorus content of cytoplasmic granules in *E. coli* expressing recombinant polyphosphate kinase is confirmed by ultrastructural and electron-loss spectroscopic analysis using energy-filtered transmission electron microscopy (EFTEM) and is increased and qualitatively altered by recombinant microcompartment co-expression**  
 A: control *E. coli* Tuner<sup>TM</sup>(DE3). B: *E. coli* Tuner<sup>TM</sup> (DE3) BMC, microcompartment only (pSF37). C: *E. coli* BL21 (DE3) P18-PPK1, targeted PPK1 (pML001+ pLysS). D,E,F,G: *E. coli* Tuner<sup>TM</sup> (DE3) P18-PPK1 + BMC, targeted PPK1 plus microcompartments (pML001 + pSF37). A-F: Electron spectroscopic imaging. Phosphorus signals are shown as overlays: green in A,B,C,F: red in D: yellow in E. Scale bar 1  $\mu$ m unless stated. G: Parallel electron

energy-loss spectroscopy (PEELS) of the largest granule in E. The red line represents SpotPEELS of the large inclusion from E with the spot (size: 16 nm) placed centrally (electron micrograph inset). The green-boxed area represents the P-L<sub>2,3</sub> energy-loss near-edge structure (ELNES), characterized by the two peaks (asterisks). The blue-coloured dashed spectrum is referenced from sodium polyphosphate.