Infection and Drug Resistance

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METHODOLOGY

Fluorescence enzymatic assay for bacterial polyphosphate kinase 1 (PPK1) as a platform for screening antivirulence molecules

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Abstract: Inorganic polyphosphate (polyP) and its metabolic enzymes are important in several cellular processes related with virulence and antibiotic susceptibility. Accordingly, bacterial polyP synthesis has been proposed as a good target for designing novel antivirulence molecules as alternative to conventional antibiotics. In most pathogenic bacteria, polyphosphate kinase 1 (PPK1), in charge of polyP synthesis from ATP, is widely conserved. Current colorimetric and radioactive polyP synthesis enzymatic assays are not suitable for high-throughput screening of PPK1 inhibitors. Given the ability of polyP to modify the excitation-emission spectra of DAPI (4′-6-diamidino-2-phenylindole), a fluorescence assay was previously developed by using a purified recombinant PPK1 enzyme from *Escherichia coli*. In this work we have developed a suitable methodology for high-throughput measurement of *E. coli* PPK1 activity. This platform can be used for the screening putative antimicrobial molecules for related enteropathogenic bacteria.

Keywords: antivirulence, antimicrobial, affinity purification, polyphosphate kinase, DAPI, fluorescence enzymatic activity

Inorganic polyphosphate (polyP) is a linear polymer ubiquitous among living organisms that presents a variety of biochemical functions. Studies of polyP-metabolizing enzymes are important for medicine and particularly infectious diseases,[1,2] because polyP functions are directly related with virulence factor production in bacteria.[3–5] In addition, mutants lacking polyP kinase 1 (PPK1), the main bacterial enzyme in charge of polyP synthesis, are more sensitive toward antibiotics.[6,7]

Given the fact that polyP synthesis is not essential for bacterial growth but for the regulation of cellular processes related to virulence, it has been proposed that polyP metabolism, and particularly polyP synthesis by the PPK1, is an attractive target for the design of novel antivirulence compounds.[8] However, a feasible polyphosphate kinase assay is currently not available for this purpose. We have therefore focused on developing a high-throughput fluorescence-based PPK1 enzymatic assay to facilitate the screening of putative inhibitor that could be used as antivirulence molecules.

For this, we took advantage that, when excited at 415 nm, fluorescence emitted by DAPI-polyP complex is detected at 550 nm and fluorescence emission from free DAPI and DAPI-DNA complex are minimal at this wavelength. In addition, the highly specific DAPI-polyP complex can be detected at polyP concentration as low as 25 ng/ml.[9]

First, *ppk1* gene of *E. coli* K12 was synthesized into pUC57, using Genescript (Piscataway, NJ, USA) service. In-frame insertion of an amino-terminal 6xHis-tag were included by cloning the NdeI/HindII fragment of the synthetic *ppk1* gene into

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pET-TEV for affinity purification purpose.[10] The resulting plasmid pET-TEV:Ec*ppk1* was transformed into *E. coli* strain BL21-AI strain for protein overexpression. Transformant clones were randomly selected for growing and induction of the recombinant protein (Figure 1A and B).

For PPK1 purification, *E. coli* BL21-AI cells transformed with the derived plasmid (pET-TEV:Ec*ppk1*) were aerobically grown in Luria-Bertani (LB) medium at 37 °C and stopped after 2 hrs of induction with L-arabinose (0.4%). As shown in Figure 1A, an induced 80 kDa polypeptide was detected in both the soluble and insoluble fractions of two clones (Figure 1A). To obtain the purified PPK1, soluble fraction of bacterial cells obtained from large-scale cultures (500 mL) were used for further protein purification (Figure 1B). For this, cells were harvested by centrifugation and resuspended in B-PER™ Bacterial Protein Extraction Reagent (Life Technologies, Carlsbad, CA, USA). The soluble cellular fraction was loaded into a HisTrap column (GE Healthcare, Chicago, IL, USA) previously charged with $NiSO_4$ (Merck, Kenilworth, NJ, USA), according to the instructions of the manufacturer. The bound proteins were eluted using a buffer Tris 50 mM MgCl2 10 mM pH 8.2 supplemented with stepwise increasing concentrations of imidazole from 40 mM to 600 mM. The eluted fractions were pooled and the purity and identity of the recombinant PPK1 were confirmed by SDS-PAGE and Western-blot, respectively (Figure 1C and D). Purified recombinant PPK1-His6-Tag was dialyzed with buffer D (20 mM Tris-HCl pH 8.2, 150 mM NaCl, 10 mM β-mercaptoethanol, 20% glycerol, 0.05% Tween 20, 50 mM ammonium sulfate and 10 mM $MgCl_2$) and was kept at −20 °C until use. The purified PPK1 were then tested in vitro for polyP synthesis assays using DAPI (Figure 2).

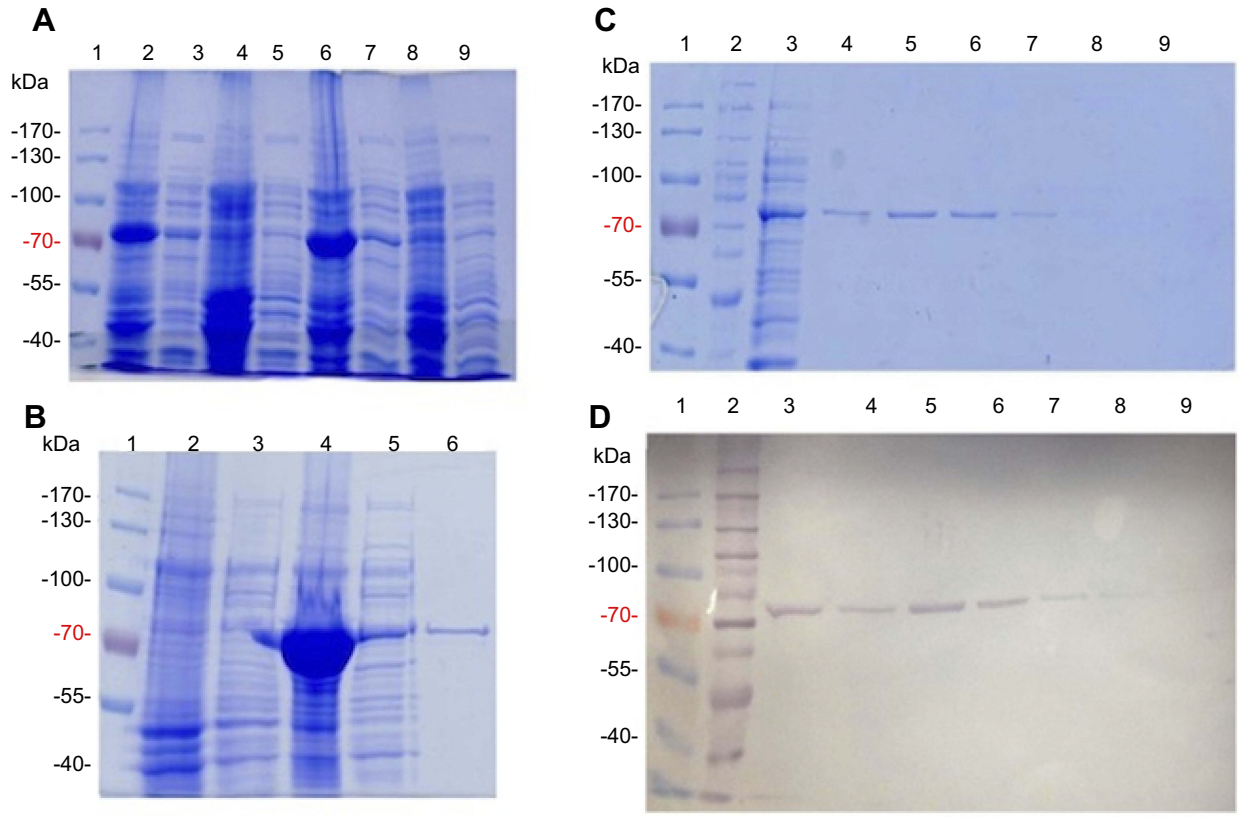
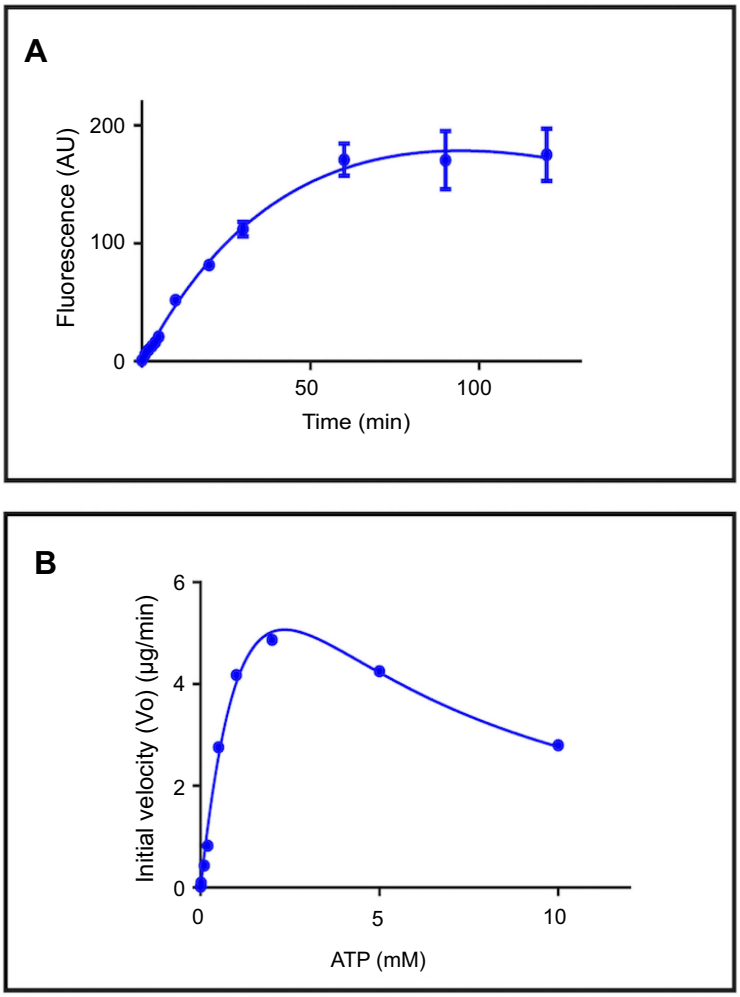


Figure 1 Purification of *E. coli* PPK1-His6- tag by immobilized metal affinity chromatography (IMAC). Recombinant *E. coli* PPK1-His6-tag was purified by immobilized Ni-Sepharose affinity chromatography as previously reported.[8] Bacterial Protein Extraction Reagent B-PER® (Thermo Fisher Scientific, Waltham, MA, USA) was used for obtaining the recombinant proteins from the bacterial pellet. (**A**) Insoluble and soluble fractions of cell proteins respectively from 4 different colonies (lanes 2–9) were obtained by centrifugation and visualized in Tris/Glycine 12% SDS–PAGE stained with Coomassie Blue. (**B**) Insoluble fraction of selected colony (line 4) was discarded and the respective soluble fraction (line 5) was equilibrated and loaded onto the affinity column (His-Trap ®; GE Healthcare, Chicago, IL, USA) to obtain the purified PPK1-His6-tag protein (line 6). Insoluble and soluble protein extracts from negative expression control were loaded into lines 2 and 3. (**C**, **D**) Fractions were eluted with 200 mM de imidazole and were analyzed by Tris/Glycine 12% SDS–PAGE. Non-retained fraction (line 3) and eluted fraction (lines 4–9) were stained with Coomassie Blue (**C**) and also by Western-Blot (**D**) using Nickel-HRP, HisDetector™ kit (KPL Inc., Milford, MA, USA).



V_{max}	Km	K_{cat}	Specific activity (1mM of ATP)
0,07 µmol/min	2,4 mM	0,24 s^{-1}	11,3µmol/min/mg

Figure 2 Polyphosphate kinase fluorescence enzymatic assay using DAPI. (**A**) PolyP kinase activity was measured using DAPI to quantify polyP production. Purified *E. coli* PPK1-His6-tag (16,5 µg/ml) was added to the reaction mix: 50 mM Hepes-KOH pH 7.2, 5 mM $MgCl_2$, 50 mM ammonium sulfate, 1 mM ATP, 1 µg/ml $poliP_{45}$, 2 mM phosphate creatine and 20 µg/ml creatine kinase. At each time reaction was stopped by adding 50 µM DAPI to 20 µL aliquots and fluorescence was read using Synergy™ Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA) using excitation and emission filters of 400/10 nm and 540/25 nm respectively. (**B**) PPK activity of the purified PPK1-His6-Tag was assayed at different substrate (ATP) concentrations.

PolyP kinase activity was assessed by incubating the purified enzyme (5 µg protein/reaction) in assay buffer (50 mM HEPES-KOH, pH 7.5 40 mM ammonium sulfate, 4 mM magnesium sulfate, 60 mM creatine phosphate and 50 µg of creatine kinase) containing 5 mM ATP as substrate (total reaction volume =200 µl) during 1 h at 37 °C.[11] This was followed by addition of DAPI at a final concentration of 50 µM. Fluorescence intensity measurements of DAPI-polyp complex were obtained using Synergy 2 Multi-Mode microplate reader (Biotek, Winooski, VT, USA) at an excitation wavelength of 400 nm (bandwidth of 10 nm). Fluorescence emission was measured at 540 nm (bandwidth of 25 nm). All experiments were performed using black fluorescence 96 well microplates and the values obtained were corrected by subtracting the fluorescence emitted by control wells containing all the ingredients, except the enzyme. Standard curves were prepared with known concentrations (1–10 µg) of poly-P_{45} (Merck) and the PPK activity was expressed as µg of polyP produced per min. (Figure S1). Similar to the PPK radioactive assay, which requires an ATP regeneration system (RS), our

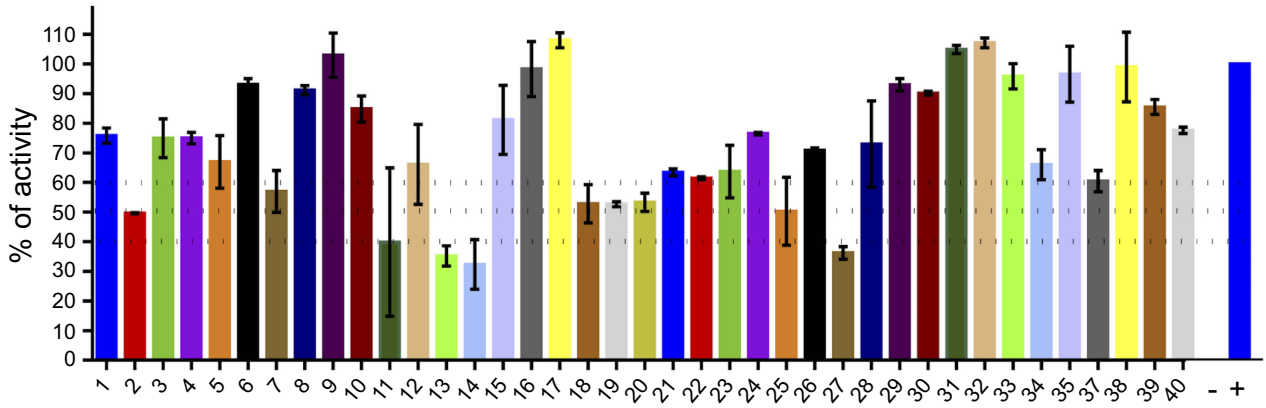


Figure 3 Screening for PPK1-His6-tag inhibitors. Inhibitors of *E. coli* PPK1 activity were selected by measuring polyP production using DAPI fluorescence. Evaluated molecules (1 μM) were previously assayed toward PPK1 from *P. aeruginosa* PAO1 (PaPPK1).[8] These compounds were selected by virtual screening for inhibitors of PPK1 and for antivirulence activity toward *P. aeruginosa* PAO1.

DAPI fluorescence assay also requires the use of creatine kinase based regeneration system (Figure S2). As shown in Figure 2, kinetic parameters from *E. coli* PPK1 by using our assay were in agreement with those previously reported with radioactive assays.[11,12]

Finally, we evaluated 40 compounds that had been previously selected by virtual screening as inhibitors of PPK1 from *P. aeruginosa* PAO1 (PaPPK1).[8] The PPK1 activity was assayed in 96-well by measuring the polyP-DAPI fluorescence at 550 nm. As shown in Figure 3, we have identified 9 compounds that inhibit around or more than 50% of *E. coli* PPK1 activity at 1 μM. Similar results for these compounds were obtained against PPK1 from *P. aeruginosa* PAO1.[8]

In conclusion, we have developed a suitable PPK assay based on polyP-DAPI fluorescence emission. The assay was successfully validated using PPK1 from *E. coli*, which was obtained by affinity purification. The purified PPK1 was used for the screening of inhibitor molecules that can be tested in future antivirulence assays. This study will serve as a basis for the screening putative antimicrobial molecules for related enteropathogenic bacteria.

Highlights

- We have synthetized and overexpressed PPK1 from *Escherichia coli* PPK1 fused to polyhistidine tag.
- A novel fluorescence PPK1 enzymatic assay was developed using DAPI.
- The fluorescence assay was used for screening of polyP synthesis inhibitors.
- The assay will be useful in high-throughput screening of putative antivirulence molecules targeting enteric pathogens.

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Disclosure

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Supplementary materials

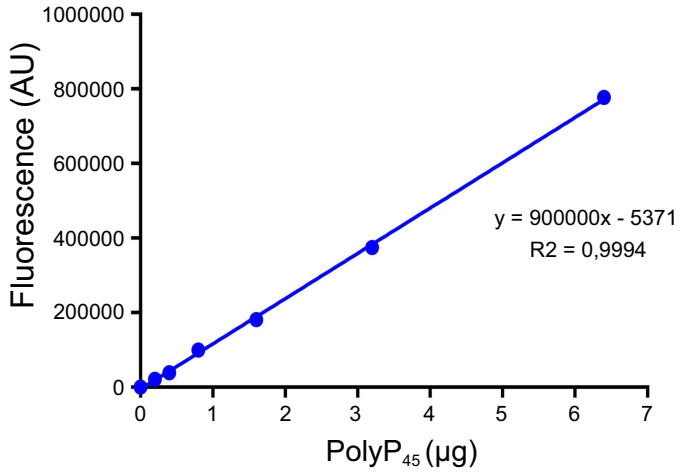

Figure S1 Calibration curve of DAPI-polyP fluorescence. Calibration curve equation was used to quantify the amount of polyP produced during the DAPI-polyP enzymatic assay.

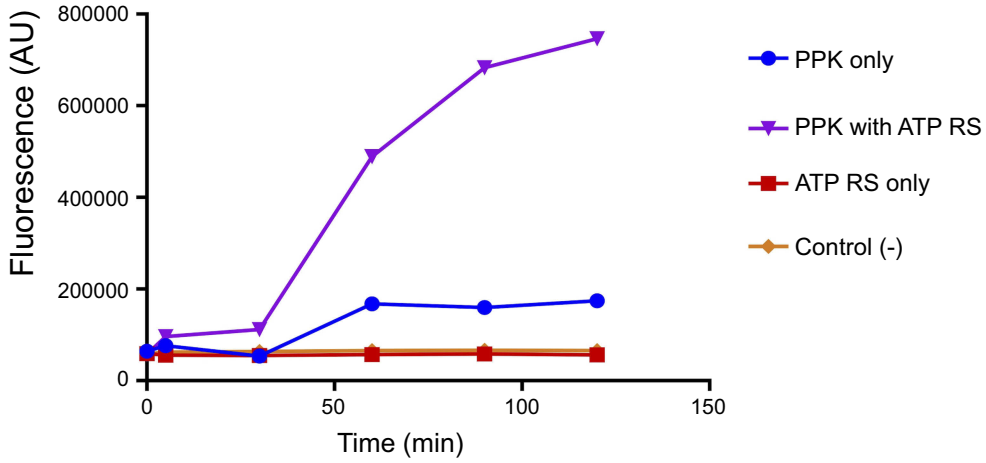

Figure S2 ATP regeneration system (RS) is essential for DAPI-polyP enzymatic assay. PPK1 activity was assay using DAPI-polyP fluorescence in the presence or absence of RS (60 mM creatine phosphate and 50 µg of creatine kinase).