Characterization of *Agrobacterium tumefaciens* PPKs reveals the formation of oligophosphorylated products up to nucleoside nona-phosphates

Celina Frank¹ · Attila Teleki² · Dieter Jendrossek¹

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**Abstract**

*Agrobacterium tumefaciens* synthesizes polyphosphate (polyP) in the form of one or two polyP granules per cell during growth. The *A. tumefaciens* genome codes for two polyphosphate kinase genes, *ppk1AT* and *ppk2AT*, of which only *ppk1AT* is essential for polyP granule formation in vivo. Biochemical characterization of the purified PPK1AT and PPK2AT proteins revealed a higher substrate specificity of PPK1AT (in particular for adenine nucleotides) than for PPK2AT. In contrast, PPK2AT accepted all nucleotides at comparable rates. Most interestingly, PPK2AT catalyzed also the formation of tetra-, penta-, hexa-, hepta-, and octa-phosphorylated nucleosides from guanine, cytosine, deoxy-thymidine, and uridine nucleotides and even nona-phosphorylated adenosine. Our data—in combination with in vivo results—suggest that PPK1AT is important for the formation of polyP whereas PPK2AT has the function to replenish nucleoside triphosphate pools during times of enhanced demand. The potential physiological function(s) of the detected oligophosphorylated nucleotides await clarification.

**Key points**

- PPK1AT and PPK2AT have different substrate specificities,
- PPK2AT is a subgroup 1 member of PPK2s,
- PPK2AT catalyzes the formation of polyphosphorylated nucleosides

**Keywords** Polyphosphate · Polyphosphate kinase · Agrobacterium tumefaciens · Nucleotides

**Introduction**

Polyphosphate (polyP) is an inorganic polymer in which phosphate residues are linked by energy-rich phosphoanhydride bonds. It can be formed either abiotically (vulcanism) or biologically by the action of polyP kinases (PPKs in prokaryotes) or other enzymes (in eukaryotes). PolyP is ubiquitously distributed in species of all domains of life (Komberg et al. 1999; Rao et al. 2009; Kulakovskaya et al. 2014). In most prokaryotes, polyP is present in form of granule-like inclusions (polyP granules or volutilin granules) with diameters mostly in the range of 50 to 200 nm. In yeasts, polyP can be accumulated up to ≈ one quarter of the cellular weight in vacuole-like compartments (Christ et al. 2020a). PolyP represents a reservoir of phosphorous and apparently is also involved in various forms of stress resistance such as tolerance against heavy metals, elevated temperature, or reactive oxygen species and can fulfill functions in virulence, motility, biofilm formation, and cell cycle control (Rashid and Kornberg 2000; Rashid et al. 2000; Nikel et al. 2013; Chuang et al. 2013; Gray and Jakob 2015; Racki et al. 2017). In humans, polyP participates in blood coagulation but is also involved in neurodegenerative diseases (Lempart and Jakob 2019).

In prokaryotes, two types of PPKs are known to catalyze the reversible formation of polyP from ATP: PPK1 type PPKs have a molecular mass of ≈ 80 kDa and consist of an N-terminal (N)
domain, a head (H) domain, and two C-terminal domains (C1 and C2) (Ahn and Kornberg; Zhu et al.). The E. coli polyP kinase is the first described PPK and the prototype of type 1 PPKs (Ahn and Kornberg). PPKs of the PPK2 type have roughly only half of the molecular masses of PPK1s (MW around 40 kDa) and are divided into three subtypes dependent on their substrate specificities for nucleoside di-phosphates (subtype 1), nucleoside mono-phosphates (subtype 2), or both (subtype 3) (Motomura et al.). Bacteria can have only the PPK1 type of PPKs, only the PPK2 type of PPKs, or both types of PPKs (PPK1 and PPK2) (Rao et al.). Several recent reports describe that some PPK2s are able to form nucleotides with four (Mordhorst et al.; Ogawa et al.; Hildenbrand et al.) or even five (Mordhorst et al.) phosphate residues.

The β-proteobacterium Ralstonia eutropha (Cupriavidus necator) is somehow special as its genome has seven ppk genes. Indeed, two PPK1s (PPK1a and PPK1b) as well as five PPK2s (PPK2a–PPK2d) have been identified in R. eutropha (Tumlirsch et al.). One of the PPK2s, PPK2c, turned out to be highly active in vitro and catalyzed the formation of microscopically detectable polyP granules in vitro (Hildenbrand et al.; Hildenbrand et al.). The other PPKs of R. eutropha have not yet been investigated. The α-proteobacterium Agrobacterium tumefaciens has two ppk genes (Atu0418 and Atu1144) that encode a type 2 PPK (PPK2AT) and a type 1 PPK (PPK1AT), respectively. In our recent study (Frank and Jendrossek), we showed that polyP in Agrobacterium tumefaciens is stored in “ordinary” polyP granules and not in membrane-enclosed acidocalcisomes as previously assumed (Seufferheld et al.). In the present study, we determined the in vitro properties of purified PPK1AT and PPK2AT. It turned out that PPK2AT is rather promiscuous with respect to its nucleotide specificity but had the so far unique property to catalyze the formation of oligophosphorylated nucleotides up to nonaphosphorylated nucleotides.

### Material and methods

#### Bacterial strains, plasmids, and culture conditions

Table 1 lists all used bacterial strains and plasmids of this study. A. tumefaciens C58 was the source of ppk genes. Genomic DNA was used to clone ppk1 (Atu1144, hereafter ppk1AT) and ppk2 (Atu0418, hereafter ppk2AT). Escherichia coli JM109 and E. coli BL21(DE3)/pLysS (Novagen) served as hosts in cloning procedures and gene expression, respectively. Cloning of ppk1AT into the pET22b expression vector (ppk1AT-his6) was done with BamHI and SacI as restriction and cloning sites. PPK2AT was cloned into pET28a (hisG-ppk2AT) with NcoI and BamHI. The pET22b-ppk1AT and pET28a-ppk2AT constructs were transformed into E. coli JM109, verified by PCR amplification and sequencing, before finally transformed into the expression strain E. coli BL21(DE3)/pLysS.

| Strain/plasmid        | Relevant characteristics                                      | Reference                  |
|-----------------------|--------------------------------------------------------------|----------------------------|
| Escherichia coli JM109| Cloning strain                                               | DSMZ3423                   |
| E. coli BL21(DE3)/pLysS| Wild type, Km<sup>+</sup>                                      | Novagen                    |
| Agrobacterium tumefaciens C58| Gene deletion of ppk1AT(atu1144) and ppk2AT(ato0418), polyP-deficient, Km<sup>+</sup> | Goodner et al. (2001) |
| A. tumefaciens C58 Δppk1 Δppk2| Gene deletion of ppk1AT(atu1144) and ppk2AT(ato0418), polyP-deficient, Km<sup>+</sup> | Frank and Jendrossek (2020) |
| pBBR1MCS2::PphaC-eyfp-c1| Broad host range vector for construction of gene fusions with eyfp, confers Km<sup>+</sup>, constitutive expression from PphaC | Pfeiffer et al. (2011) |
| pBBR1MCS2::PphaC-mCherry-n1| Broad host range vector for construction of gene fusions with mCherry, confers Km<sup>+</sup>, constitutive expression from PphaC | Pfeiffer et al. (2011) |
| pBBR1MCS2::PphaC-ppk1AT-mCherry| Constitutive expression of ppk1AT-mCherry (atu1144) | This study |
| pBBR1MCS2::PphaC-eyfp-ppk2AT| Constitutive expression of eyfp-ppk2AT | Frank and Jendrossek (2020) |
| Ralstonia eutropha H16| Wild type, Km<sup>+</sup>                                      | DSMZ428                    |
| R. eutropha H16 Δppk-all| Chromosomal deletion of Δppk1a, Δppk1b, Δppk2a, Δppk2b, Δppk2c, Δppk2d, Δppk2e (A0104, B1019, A0226, A0997, A1212, A0997, A1271, A1979), Km<sup>+</sup> polyP deficient | Tumlirsch and Jendrossek (2017) |

Kanamycin resistance (Km<sup>+</sup>) and sensitivity (Km<sup>+</sup>)
Purification of PPK1<sub>AT</sub> and PPK2<sub>AT</sub> and PPK activity assays

Purifications of PPK1<sub>AT</sub> with a C-terminal and PPK2<sub>AT</sub> with an N-terminal hexa-histidine tag were done as previously described for PPK2c of Ralstonia eutropha (Hildenbrand et al. 2019). Purified PPK proteins (1–4 mg/ml) were stored on ice directly in elution buffer (pH 8.0 (composition see below) without any further treatment (250 mM imidazole, 300 mM NaCl and 50 mM NaH<sub>2</sub>PO<sub>4</sub>). PPK activity assays and reaction product determination via HPLC were performed as described in detail previously (Hildenbrand et al. 2020).

Reaction conditions for the formation of oligophosphorylated nucleosides by PPK2<sub>AT</sub> and HPLC-MS/MS analysis

Samples containing 1 μM enzyme, 15 mM NTPs, 2 mM MnCl<sub>2</sub>, and 0.1 M Tris-HCl buffer (pH 7–8) were incubated for times as indicated (30 min up to 48 h at 30 °C). Alternatively, 1 μM PPK2<sub>AT</sub> was incubated with 2 mM NDP (or dTDP [TDP not available]) and 9 mM polyP in assay buffer (0.1 M Tris-HCl buffer, pH 8, 2 mM MnCl<sub>2</sub>) for times as indicated. The reactions were terminated by heating of the assay mixture to 95 °C for 3 min. For later use, the samples were stored at −20 °C or measured directly. Samples were thawed on ice and centrifugated for 15 min at 20,000 g and 4 °C. Targeted LC-MS measurements were performed with enhanced sensitivity on an Agilent 1200 HPLC system coupled with an Agilent 6410B triple quadrupole mass spectrometer (QQQ). Sample preparation and chromatographic separation of nucleoside phosphates by bicratic polymer-based zwitterionic hydrophilic interaction chromatography (ZIC-pHILIC) was strongly enriched and PPK2<sub>AT</sub> (36.2 kDa) was almost homogeneous.

Biochemical characterization of PPK1<sub>AT</sub>

The substrate specificity of PPK1<sub>AT</sub> was analyzed by incubation of PPK1<sub>AT</sub> with ATP, GTP, CTP, dTTP, or UTP for 30 min and subsequent determination of the respective nucleotide compositions by HPLC. It turned out that the activity of PPK1<sub>AT</sub> with NTPs was hardly detectable if low concentrations of NTPs (2 mM) were used. Only at high concentrations of the NTPs (15 mM) and after 24 h reaction time polyP-forming activity was detected as revealed by formation of NDPs at the expense of NTPs (Online resource 2). The highest activity was determined with the purines ATP and GTP. With GTP as substrate (but not with other NTPs), the formation of polyP was shown by gel electrophoresis and subsequent staining with toluidine blue (Online resource 3). Presumably, the concentration of formed polyP by the other nucleotides was too low to be detected by toluidine staining.

Toluidine staining of polyP after polyacrylamide gel electrophoresis

Enzymatically produced polyP (by (PPK1<sub>AT</sub> or PPK2<sub>AT</sub>) was separated by electrophoresis in 15% poly acrylamide gels and stained with toluidine blue according to (Losito et al. 2009). A synthetic polyP standard with ≈ 100 P<sub>i</sub> residues in average (gift of A. Saidari) was used as standard. PolyP concentrations in this contribution always refer to the concentration of the monomeric phosphate (P<sub>i</sub>).

PolyP quantification

The amount of polyP was quantified after polyP extraction followed by digestion to phosphate with exopolyphosphatase and subsequent phosphate determination (malachinte green assay) as recently summarized in (Christ et al. 2020b).

Results

Purification of polyP kinases

The two polyP kinase genes of A. tumefaciens (ppk1<sub>AT</sub>) and (ppk2<sub>AT</sub>) were PCR amplified, cloned in fusion with a hexa-histidine-coding sequence into pET22b or pET28a, respectively, and expressed in E. coli BL21. PPK1<sub>AT</sub> and PPK2<sub>AT</sub> eluted during nickel agarose affinity chromatography of soluble cell extracts at approximately 250 mM imidazole. PPK1<sub>AT</sub> and PPK2<sub>AT</sub> containing fractions were combined, concentrated, and tested for purity on a polyacrylamide gel (Online resource 1). PPK1<sub>AT</sub> (84.9 kDa) was strongly enriched and PPK2<sub>AT</sub> (36.2 kDa) was almost homogeneous.
NTP-synthesizing activities were detected for the other nucleotides (GDP, CDP, UDP, or dTDP). Only by elongation of the assay time to 6 h the formation of other NTPs from the respective NDPs and polyP became detectable (Fig. 1b). The formation of adenosine tetraphosphate (AP4), however, was not detected (data not shown). AP4 and related NP4s had been previously detected as reaction products of PPKs in earlier studies (see “Discussion” for details and references).

**Biochemical characterization of PPK2 AT** Purified PPK2 AT (as PPK1 AT) was not able to phosphorylate NMPs with polyP as phosphate donor (data not shown) and therefore is a member of subgroup 1 of PPK2s according to the PPK2 classification of Motomura et al. (Motomura et al. 2014).

PPK2 AT catalyzed the formation of polyP from ATP (NTP) as evident from the decrease of the ATP (NTP) concentration and a concomitant increase of the ADP (NDP) concentrations in PPK assays with purified PPK2 AT after an incubation period of 30 min (Online resource 4). When the reaction products were gel-electrophoretically separated and stained with toluidine blue, strong staining signals were detected as a pink smear and indicated that a mixture of long-chain polyP molecules had been formed (Fig. 3, Online resource 5a). In these experiments, Mn$^{2+}$ ions were used as cofactor. However, Mn$^{2+}$ could be replaced by Mg$^{2+}$ and by Ca$^{2+}$ but not by monovalent ions such as Na$^+$ or K$^+$. Poor activity of PPK2 AT was detected in the presence of Co$^{2+}$ (Fig. 2b).

Similar results were obtained when PPK2 AT was incubated with GTP or UTP (instead of ATP) but not with CTP or dTTP as substrates for 30 min. However, after longer incubation time, polyP-toluidine signals became detectable also for dTTP (2 h) and for CTP (24 h) (Fig. 3, Online resource 5b). Interestingly, the polyP-toluidine signals that were clearly detectable after 30 min for GTP, dTTP, and UTP as substrates vanished after longer incubation times; instead, nucleoside phosphates with more than three phosphate residues were detected by HPLC from GTP, dTTP, and UTP (see next chapter for details). This indicated that polyP was used as an intermediate substrate for the synthesis of guanosine-, d-thymidine-, or uridine-oligophosphates in case of the reactions with GTP, dTTP, and UTP. In contrast, even after 48 h of incubation, the polyP-toluidine signals produced from ATP and CTP were still present and no oligophosphorylated

![Fig. 1](image1.png) **Enzyme activity of PPK1 AT.** The reactions were performed in the direction of NTP synthesis from NDPs (2 mM) and polyP (9 mM P$_i$) and in the presence of 2 mM MnCl$_2$. a Relative activity (%) after 30-min incubation. b Relative activity (%) after 6-h incubation. Assays were performed in triplicates; error bars indicate standard deviations.

![Fig. 2](image2.png) **Enzyme activities of PPK1 AT and PPK2 AT with different cations.** Activities of PPK1 AT (a) in polyP synthesis direction (from 2 mM ATP, 30 min reaction time) and of PPK2 AT (b) in NTP synthesis direction (from 2 mM ADP, 9 mM polyP, 30-min reaction time) with different cations (2 mM) were determined. The activities were maximal with CaCl$_2$ (PPK1 AT, 0.01 μmol ATP/min/mg expressed as average of the first 30 min) or with MgCl$_2$ (PPK2 AT, 0.09 μmol ADP/min/mg) expressed as average of the first 30 min, respectively, and were taken as 100%. Assays were performed in triplicate; error bars indicate standard deviations.
nucleotides (except NP4) were detected with ATP and CTP by HPLC and UV-vis detection (for overview see Online resource 6).

PPK2AT phosphorylates NDPs with polyP as phosphor donor and produces NTPs and nucleoside oligophosphates. The identification of HPLC signals after prolonged incubation of some NTPs with PPK2AT that most likely corresponded to oligophosphorylated nucleosides prompted us to investigate whether these assumed oligophosphorylated nucleosides were also formed from NDPs in the presence of polyP. To this end, purified PPK2AT was incubated with ADP, GDP, CDP, dTDP, or UDP (each 2 mM) in the presence of 9 mM polyP for 24 h. Analysis of the products by HPLC revealed that the concentrations of the respective NDPs were substantially reduced (Fig. 4a) and the concentrations the corresponding NTPs were increased. Additional peaks appeared in the HPLC chromatograms of all tested NDPs at higher retention time than that of the respective NTP in each assay and corresponded to adenosine tetraphosphate (AP4), guanosine tetraphosphate (GP4), cytosine tetraphosphate (CP4), desoxy-thymidine tetraphosphate (dTP4), and uridine tetraphosphate (UP4) (Online resource 6) as it has been previously shown for other PPK2s. Apparently, PPK2AT is a member of the growing
group of PPK2s that catalyzes the formation of nucleoside tetraphosphates from NDPs and polyP (see below for more details and references). Furthermore, the HPLC chromatograms of the PPK2AT assays showed even more additional peaks at higher retention times than the respective NP4s for the reactions with GDP, CDP, dTDP, and UDP (exemplarily shown for the reaction with GDP and polyP in Fig. 4b, all other chromatograms in Online resource 6). Assuming that each peak at higher retention time than the respective NTP corresponds to molecules with one more phosphate group, this would mean that PPK2AT can catalyze the formation of guanosine oligophosphates (GP4-GP8), cytosine oligophosphates (CP4-CP8), deoxy-thymidine oligophosphates (TP4-TP8), and uridine oligophosphates (UP4-UP7). The retention times of these additional peaks were identical to the retention times of the additional peaks that had been detected after prolonged incubation of PPK2AT with GTP, dTTP, or UDP (see above). We assume that these peaks represent nucleotides with a higher number of phosphate residues than four (see Online resource 7 for summary). Nucleotides with four and five phosphate residues have been previously described as reaction products of PPKs (Mordhorst et al. 2019) but higher phosphorylated nucleosides have not yet been described.

Identification of nucleoside oligophosphates by ZIC-pHILIC-MS/MS

To verify that the observed products really represent the postulated oligophosphorylated nucleosides, the products of the PPK2AT-catalyzed reaction with NTPs were analyzed by HPLC and HPLC-MS/MS. Figure 5 shows the products of the reaction of PPK2AT with NTPs separated by HPLC. Again, peaks appeared at higher retention times than the corresponding NTPs and were identified as oligophosphorylated nucleotides by ZIC-pHILIC-MS/MS. Notably, the deduced masses perfectly matched the expected masses of guanosine tetra-, penta-, hexa-, hepta-, and octa-phosphate each with mass increments of 80 (corresponding to one phosphate unit in polyP; see Table 2). The MS detector even detected molecules with m/z values that corresponded to guanosine nona-phosphate (GP9) and guanosine deca-phosphate (GP10), however, with insufficient signal-to-noise ratios. The signal-to-noise ratios were too low for GP9 and GP10 to allow a reliable identification and the concentrations of the higher phosphorylated nucleosides were too low to be visible in the HPLC UV-vis detection. Analog results were obtained for the products of PPK2AT with the other nucleotides (ATP, GTP, CTP, dTTP, and UTP). With each nucleotide, m/z signals for up to the octa-phosphorylated nucleosides (and up to the nona-phosphorylated nucleotide in case of ATP) were clearly detected by ZIC-pHILIC-MS/MS. For a summary of all identified nucleotides, see Table 2 and Online resource 7 and 8. In summary, PPK2AT catalyzes the transfer of multiple P1 residues from polyP to NDPs with up to the nona-phosphorylated nucleosides as products. Presumably, nucleotides with even more phosphate residues than eight will become detectable by variation of the assay parameters and/or optimization of the MS detection parameters.

Expression of ppk1AT and ppk2AT in polyP-deficient backgrounds

To test the function of the A. tumefaciens ppk genes in vivo, we expressed the ppk1AT and ppk2AT genes as fusions with the enhanced yellow fluorescent gene (eYFP) (or with mCherry) in a polyP-deficient background of A. tumefaciens (Δppk1AT, ppk2AT) and R. eutropha (Δppk-all, with all seven ppk genes deleted). Expression of ppk1AT restored the formation of polyP granules in A. tumefaciens (Δppk1AT, ppk2AT) (Online resource 9) and confirmed that PPK1AT is able to form polyP in vivo. A similar result was obtained when ppk1AT was expressed in R. eutropha (Δppk-all) (Fig. 6b). To our surprise, the expression of ppk2AT also resulted in restoration of polyP granule synthesis in the polyP-deficient R. eutropha background (Fig. 6c) but not in A. tumefaciens (Δppk1AT, ppk2AT) (Online resource 9). These results confirmed that both PPKs of A. tumefaciens principally are able to catalyze the formation of polyP in vivo. In most cells, the formed fusion proteins (eYFP-PPK2AT) colocalized with DAPI-stained polyP granules in both species. However, PPK1AT-mCherry signals were elliptically shaped and were located near the cell pole in A. tumefaciens sometimes—but not always—in close proximity to a formed polyP granule (Online resource 9). In R. eutropha (Δppk-all), the same construct was distributed more or less homogenously in the whole cell around the polyP granules (Fig. 6b), but did not colocalize with them.

Discussion

This activity and substrate specificity determinations in our study showed that PPK1AT is a typical type 1 PPK (Rao et al. 2009) having a preference (or high affinity) for adenine nucleotides and that PPK2AT is a member of the subtype 1 group of PPK2s with specificity for NDPs but not for NMPs as it had been previously postulated by Motomura et al. (2014) from amino acid sequence analysis. PPK2AT has the so far unique property to transfer multiple phosphate groups to GDP, dTDP, or UDP (and to lower extent also to ADP and CDP) and to form oligophosphorylated products up to the octa-phosphorylated nucleosides or up to AP9 in case of adenine nucleotides. So far, only tetra- or penta-phosphorylated
nucleotides have been detected as reaction products of PPK2 of *Meioccoccus ruber* (Mordhorst et al. 2019). Using *A. tumefaciens* PPK2AT, the oligophosphorylated nucleosides were formed from the diphosphates in the presence of polyP immediately. In contrast, it took much longer until oligophosphorylated nucleosides became detectable if PPK2AT was tested in the direction of polyP synthesis from NTPs: it was first necessary that polyP molecules were formed before up to the deca-phosphorylated nucleosides became detectable. This finding suggests that PPK2AT catalyzes the transfer of short phosphate chains from polyP to NDPs. A multiple transfer of single phosphate residues from polyP to NDPs is also possible. The reaction worked best with GDP, dTDP, or UDP and with lower efficiency also with ADP (up to AP9).

### Table 2

Results and parameters of HILIC-QQQ-MS/MS measurements of nucleotide species in PPK2AT assays and standard mixtures

| Nucleotides | MS/MS parameters (SIM) | HILIC-QQQ-SIM results | HPLC results |
|-------------|------------------------|------------------------|--------------|
|             | Polarity | Species | m/z (u) | Area (Cts) | t_R* (min) | Area (Cts) | t_R* (min) | t_R* (min) |
| GMP         | [-]      | [M-H]-  | 362    | 1.89E+07 | 17.63     | 6.82E+07 | 17.59     | 3.1 |
| GDP         | [-]      | [M-H]-  | 442    | 6.43E+08 | 19.27     | 1.73E+08 | 19.33     | 3.3 |
| GTP         | [-]      | [M-H]-  | 522    | 4.64E+08 | 20.64     | 1.58E+08 | 20.76     | 4.0 |
| GP4         | [-]      | [M-H]-  | 602    | 2.39E+08 | 22.16     |          | 4.5      |      |
| GP5         | [-]      | [M-H]-  | 682    | 3.55E+07 | 23.82     |          |          | 5.2 |
| GP6         | [-]      | [M-H]-  | 762    | 5.49E+06 | 25.32     |          |          | 5.8 |
| GP7         | [-]      | [M-H]-  | 842    | 5.84E+05 | 26.84     |          |          | 6.3 |
| GP8         | [-]      | [M-H]-  | 922    | 7.31E+04 | 27.97     |          |          | 6.7 |
| dTTP        | [-]      | [M-H]-  | 321    | < LOD**  | na***     | 1.66E+08 | 14.10     | 4.6 |
| dTDP        | [-]      | [M-H]-  | 401    | 6.76E+05 | 16.36     | 2.47E+08 | 16.35     | 5.5 |
| dTTP        | [-]      | [M-H]-  | 481    | 2.21E+07 | 18.04     | 1.84E+08 | 17.86     | 6.1 |
| dTTP        | [-]      | [M-H]-  | 561    | 4.94E+08 | 20.89     |          |          | 6.9 |
| dTTP        | [-]      | [M-H]-  | 641    | 1.08E+08 | 22.58     |          |          | 7.3 |
| dTTP        | [-]      | [M-H]-  | 721    | 5.99E+07 | 24.10     |          |          | 7.5 |
| dTTP        | [-]      | [M-H]-  | 801    | 2.03E+07 | 25.49     |          |          | 7.7 |
| dTTP        | [-]      | [M-H]-  | 881    | 7.33E+06 | 26.76     |          |          | 7.9 |
| AMP         | [-]      | [M-H]-  | 346    | 1.67E+08 | 15.09     | 1.36E+08 | 15.09     | 4.7 |
| ADP         | [-]      | [M-H]-  | 426    | 4.63E+08 | 16.69     | 1.76E+08 | 16.90     | 6.4 |
| ATP         | [-]      | [M-H]-  | 506    | 1.02E+09 | 18.44     | 1.85E+08 | 18.32     | 7.8 |
| AP4         | [-]      | [M-H]-  | 586    | 2.75E+08 | 19.96     |          |          | 8.2 |
| AP5         | [-]      | [M-H]-  | 666    | 1.66E+08 | 22.26     |          |          | 7.3 |
| AP6         | [-]      | [M-H]-  | 746    | 8.38E+06 | 23.61     |          |          | na  |
| AP7         | [-]      | [M-H]-  | 826    | 2.08E+06 | 24.82     |          |          | na  |
| AP8         | [-]      | [M-H]-  | 906    | 3.22E+05 | 26.10     |          |          | na  |
| AP9         | [-]      | [M-H]-  | 986    | 1.47E+05 | 27.27     |          |          | na  |
| UMP         | [-]      | [M-H]-  | 323    | 1.59E+06 | 16.23     | 1.08E+08 | 16.23     | 2.9 |
| UDP         | [-]      | [M-H]-  | 403    | 2.27E+07 | 17.92     | 1.98E+08 | 18.15     | 3.1 |
| UTP         | [-]      | [M-H]-  | 483    | 1.69E+07 | 19.27     | 2.35E+08 | 19.57     | 3.8 |
| UP4         | [-]      | [M-H]-  | 563    | 1.27E+07 | 20.98     |          |          | 4.4 |
| UP5         | [-]      | [M-H]-  | 643    | 1.91E+06 | 22.56     |          |          | 5.2 |
| UP6         | [-]      | [M-H]-  | 723    | 2.16E+05 | 24.10     |          |          | 5.9 |
| UP7         | [-]      | [M-H]-  | 803    | 5.75E+03 | 26.04     |          |          | 6.4 |
| UP8         | [-]      | [M-H]-  | 883    | 4.78E+03 | 27.12     |          |          | 6.8 |
| CMP         | [-]      | [M-H]-  | 322    | 3.36E+07 | 16.90     | 8.69E+07 | 16.95     | 2.6 |
| CDP         | [-]      | [M-H]-  | 402    | 6.04E+08 | 18.42     | 1.35E+08 | 18.63     | 3.0 |
| CTP         | [-]      | [M-H]-  | 482    | 6.97E+08 | 19.89     | 1.21E+08 | 20.00     | 3.7 |
| CP4         | [-]      | [M-H]-  | 562    | 7.24E+07 | 21.64     |          |          | na  |
| CP5         | [-]      | [M-H]-  | 642    | 1.50E+06 | 23.25     |          |          | na  |
| CP6         | [-]      | [M-H]-  | 722    | 4.64E+04 | 24.78     |          |          | na  |
| CP7         | [-]      | [M-H]-  | 802    | < LOD**  | na***     |          |          | na  |
| CP8         | [-]      | [M-H]-  | 882    | < LOD**  | na***     |          |          | na  |

*Retention time of the nucleotide species
**Limit of detection
***Not available

Peak areas (Cts) and HILIC retention times (min) were achieved by QQQ-SIM detection (m/z ± 0.3 u). Retention times of HPLC measurements (min) are comparatively depicted in the most right column.
with CDP (up to CP8). We do not know whether the in vitro detected oligophosphorylated nucleosides were also formed in vivo and if they were formed, what physiological function they might fulfill. The in vitro formation of oligophosphorylated nucleosides required the presence of Mn$^{2+}$ ions that are not likely to be present in millimolar concentrations in vivo. Interestingly, in *Myxococcus xanthus*, AP4 (and diadenosine tetraphosphate and diadenosine pentaphosphate) have been detected and are formed by the action of some amino acyl tRNA synthetases and by phosphoglycerate kinase (Kimura et al. 2018) suggesting that an astonishing variety of oligophosphorylated nucleotides can be formed in microorganisms in vivo.

PPK2$_{AT}$ is a rather unspecific enzyme and converts all tested NDPs to the corresponding NTPs as the main product in the presence of polyP at high rates. This suggests that the in vivo function of PPK2$_{AT}$ is to replenish lowered NTP (and/or dNTP) pools from previously accumulated polyP during phases of enhanced demand, e.g., during DNA replication or at high transcription rates. This corresponds with previous studies in our lab in which we showed that PPK2$_{AT}$ is not necessary for polyP formation and that Δppk1AT (Frank and Jendrossek 2020) deletion strains were not able to form polyP granules. Accordingly, a Δppk2AT deletion strain produced slightly more polyP than the wild type presumably because the polyP-consuming activity of PPK2$_{AT}$ is absent. The reason why PPK2$_{AT}$ was able to restore the formation of polyP granules in *R. eutropha* but not in *A. tumefaciens* (despite good in vitro polyP-forming activities of PPK2$_{AT}$) might lie in different in vivo nucleotide concentrations in both strains.
Higher in vivo concentrations of NTPs in *R. eutropha* compared with *A. tumefaciens* might allow the formation of polyP from expressed ppk2AT.

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Authors’ contributions DJ designed the study and wrote the manuscript. CF performed most experiments, analyzed the data, and prepared the “Material and method” section and the figures. AT conducted HPLC-MS/MS and analyzed the MS data. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethic approval This article does not contain any studies with human participants or animals performed by any of the authors.

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