The Multiple Roles of Polyphosphate in *Ralstonia eutropha* and Other Bacteria

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Abstract

An astonishing variety of functions has been attributed to polyphosphate (polyP) in prokaryotes. Besides being a reservoir of phosphorus, functions in exopolysaccharide formation, motility, virulence and in surviving various forms of stresses such as exposure to heat, extreme pH, oxidative agents, high osmolarity, heavy metals and others have been ascribed to polyP. In this contribution, we will provide a historical overview on polyP, will then describe the key proteins of polyP synthesis, the polyP kinases, before we will critically assess of the underlying data on the multiple functions of polyP and provide evidence that – with the exception of a P-storage-function – most other functions of polyP are not relevant for survival of *Ralstonia eutropha*, a biotechnologically important beta-proteobacterial species.

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Introduction

Historical Background

The presence of insoluble phosphate-containing inclusions in microorganisms has been known for a long time. The first descriptions go back to Babes [Babes 1885] and Liebermann [Liebermann 1888] and were first summarized by A. Meyer in 1904 [Meyer 1904]. PolyP is also known as metaphosphate or volutin granules (identification of polyP in *Spirillum volutans*) because of the meta-chromic shift of basic dyes upon binding to polyphosphate [Wiame 1947]. PolyP was regularly found in yeast [Widra 1959] and in several bacterial species such as *Aerobacter aerogenes* [Smith et al. 1954] and in particular in *Mycobacterium* and *Corynebacterium* species [Sall et al., 1958; Knaysi 1959]. It became evident that volutin (polyP) granules are widespread in microorganisms (for early reviews see [Widra 1959; Harold 1966; Kulaev and Vagabov 1983], and meanwhile it is well accepted that polyP is present in all kingdoms of life [Kornberg et al., 1999; Kulaev and Kulakovskaya 2000; Rao et al., 2009] and presumably is present in every species. For the most recent reviews on polyP see [Albi and Serrano 2016; Jiménez et al., 2016; Xie and Jakob 2018].

Functions of PolyP

Many functions have been ascribed to polyP. The most evident one is that of a storage compound for phosphorus and the counter-ions to the phosphate anions such as Ca^{2+} , Mg^{2+} , K^{+} . However, additional functions have been addressed to polyP: polyP can be a reservoir for energy due to its energy-rich phosphor-anhydride bonds and polyP can substitute ATP in polyP-dependent phosphorylation (kinase) reactions. PolyP is directly or indirectly involved in pathogenicity [Parks and Hobden 2005; Peng et al., 2016; Kumar et al., 2016; Srisanga et al., 2019] and in motility [Rashid et al., 2000; Shi et al., 2004; Zhang et al., 2005; Fraley et al., 2007; Hossain et al., 2008]. PolyP can modulate the cellular responses to various stresses and is important for the adaptation to stationary phase conditions [Rao and Kornberg 1996; Nikel et al., 2013; Casey et al., 2013; Gray and Jakob 2015]. In *Escherichia coli*, polyP is part of the stringent response to nutrient downshifts [Kulaev and Vagabov 1983; Rao et al., 1998; Kornberg et al., 1999; Kuroda et al., 1999; Kulaev and Kulakovskaya 2000; Kuroda et al., 2001], recently updated by M. Gray [Gray 2019]. PolyP may be also involved in the conversion of (pathogenic) bacteria into the viable but non culturable (VBNC) state [Gangaiah et al., 2009].

Research of the last two decades showed that polyP is involved in and important for tolerance against stresses (heat, UV light, solvents, neutrophilic oxidants/hypohalous acids, heavy metals, antibiotics, survival in stationary phase and others) [Kim et al., 2002; Fraley et al., 2007; Seufferheld et al., 2008; Varela et al., 2010; Nikel et al., 2013; Tocheva et al., 2013; Alcántara et al., 2014; Gray and Jakob 2015; Bru et al., 2017; Groitl et al., 2017; Sultana et al., 2020]. Compounds that inhibit the formation of polyP in bacteria (mesalamine = 5-amino-salicylic acid) reduce the polyP content in intestinal bacteria in mammals and in turn sensitize them to host defense by reactive oxygen species. This could explain the positive effect of mesalamine on patients suffering from ulcerative colitis [Dahl et al., 2017]. Recently published results provide evidence both for eukaryotes (yeast) and prokaryotes (*Pseudomonas aeruginosa*) that polyP is necessary for cell cycle control, cell cycle exit and repair of DNA damage [Bru et al., 2016; Racki et al., 2017; Bru et al., 2017]. PolyP has also a prominent role in bacteria that are part of the biological phosphate removal process (EBPR) in sewage sludge [Blackall et al., 2002; Seviour et al., 2003; Yang et al., 2017].

In proteins, polyP can be covalently bound to lysine residues in a process named polyphosphorylation. This posttranslational modification can modulate the protein

activity similarly as other covalent modifications [Azevedo et al., 2015; Azevedo and Saiardi 2016]. Recently, it was shown that polyP can have a chaperone-like function by binding to partially denatured/unfolded proteins and keeping them in a refolding competent state [Gray et al., 2014]. In mammals, polyP is part of the blood coagulation system (pre-activation of Hageman factor XII by binding to polyP). Furthermore, polyP can interact with proteins present in the brain of mammals. PolyP is able to bind to preforms of α -synuclein fibrils thereby preventing their uptake by neurones. α -Synuclein is a key protein in neurodegenerative diseases such as Alzheimer or Parkinson [Yoo et al., 2018; Lempart and Jakob 2019; Lempart et al., 2019] and can form amyloid-like aggregates that are typical indications occurring prior to the recognizable outbreak of such diseases. PolyP can protect the organism from the formation of pathogenic amyloid plaques by binding to partially unfolded α -synuclein proteins and preventing them from further misfolding. These findings suggest that polyP has a prominent function in neurodegenerative diseases.

PolyP Kinases Are the Key Enzymes of PolyP Metabolism in Prokaryotes

PolyP kinases (PPKs) catalyze the reversible formation of polyP by transfer of a γ -phosphate group from ATP (or another NTP) to a growing chain of polyP. The presence of an oligophosphate primer is not necessary to initiate the reaction. The first isolated and biochemically characterized PPK was that of *E. coli* [Ahn and Kornberg 1990; Kumble et al., 1996]. The PPK of *E. coli* is the prototype of so-called type 1 PPKs (PPK1s) which are proteins with molecular masses of ≈ 80 kDa and are composed of four domains (N-terminal domain (N), head domain (H), and two C-terminal domains (C1 and C2)) [Zhu et al., 2005]. A second type of PPK (so-called PPK2s) has been identified first in *P. aeruginosa* [Zhang et al., 2002; Ishige et al., 2002] and later in many other bacteria. Currently known PPK2s mostly have about half of the molecular masses of PPK1s (35–40 kDa) and are characterized by a so-called PPK2-domain. PPK2 of *P. aeruginosa* prefers the reverse reaction, i.e., the nucleotide dikinase reaction (NTP synthesis from polyP and NDPs) and works best with GDP compared to ADP and other NDPs. Therefore, PPK2 of *P. aeruginosa* might have a function to provide enough GTP for example for exopolysaccharide biosynthesis [Ishige et al., 2002] and this might be one reason for polyP being important for virulence. Phylogenetic analysis of the amino acid sequences and comparison with biochemical properties of isolated PPK2s revealed that

PPK2s can be divided into three subgroups [Motomura et al., 2014]: members of subgroup I and II catalyze the phosphorylation of nucleoside diphosphates and nucleoside monophosphates, respectively, while subgroup III PPK2s can catalyze both reactions.

Analysis of prokaryotic genome sequences revealed that bacteria can have either a *ppk1* gene, a *ppk2* gene or both a *ppk1* and *ppk2*. Many bacterial species have even multiple copies of *ppk1* and *ppk2* genes. *Ralstonia eutropha*, for example, has two *ppk1* genes (*ppk1a*, *ppk1b*) and five *ppk2* genes (*ppk2a* – *ppk2e*) [Tumlirsch et al., 2015]. The presence of multiple genes for both types of PPKs suggests that they fulfill different functions some of which presumably have not yet been identified. Biochemically characterized PPK2s have a low substrate specificity and accept both purine and pyrimidine nucleotides [Motomura et al., 2014]. PPK2c of *R. eutropha* is the most unspecific PPK among biochemically characterized PPKs and accepts all natural ribo- and deoxyribonucleotides [Hildenbrand et al., 2020]. The purified protein is even able to form microscopically detectable polyP granules in vitro from NTPs [Hildenbrand et al., 2019]. Some PPKs, in particular PPK2s, not only form NTPs from polyP and NDPs but also catalyze the formation of oligo-phosphorylated nucleosides with more than three phosphate units. The first example was PPK1 of *E. coli* that catalyzed the formation of guanosine tetraphosphate from GDP and polyP [Kuroda and Kornberg, 1997]. In 2019, Mordhorst et al. showed that the PPK2s of *Meiococcus ruber*, *Sinorhizobium meliloti*, *Francisella tularensis* and *Acinetobacter johnsonii* formed tetra- and penta-phosphorylated adenosine from ADP and polyP [Mordhorst et al., 2019]. Very recently, another PPK2, PPK2 of *Agrobacterium tumefaciens*, was found to catalyze the formation of highly phosphorylated nucleosides up to the nona-phosphates from any NDP in the presence of polyP [Frank et al., 2020]. The physiological functions, that these oligophosphorylated nucleosides might have, are not known.

PolyP Metabolism in *R. eutropha*

R. eutropha strain H16 (alternative designation *Cupriavidus necator*) is a famous β -proteobacterium due to its ability to grow chemolithoautotrophically (H_2/CO_2) and to accumulate large amounts of the “bioplastic” poly(3-hydroxybutyrate) (PHB) [Pohlmann et al., 2006]. Another interesting property of *R. eutropha* is the presence of seven *ppk* genes in its genome. At least three PPKs, PPK1a, PPK2b and PPK2c, contribute to the biosynthesis of polyP granules and four of them (PPK1a, PPK2c, PPK2d and PPK2e) are associated with polyP granules in vivo as re-

Fig. 1. Model of a polyP granule in *R. eutropha*. Proteins attached to polyP are symbolized as spheres with proposed designations. The association of four polyP kinases (PPK1a, PPK2c, PPK2d, PPK2e), of two CHAD-motif containing proteins (phosins PptA, PptB) and of two additional proteins (PPI18, PPI27) with polyP granules has been demonstrated in vivo. Putative counter-ions (Mg^{2+} , Ca^{2+} , Mn^{2+} , Mn^{2+} , K^{+}) of negatively charged polyP molecules are also indicated. The presence of Mn^{2+} ions in vivo is speculative but – in vitro – it has been demonstrated, that the presence of Mn^{2+} ions effectuates the formation of oligo-phosphorylated nucleotides by PPK2c [Hildenbrand et al., 2020]. The PPK kinases PPK1b, PPK2a and PPK2b are not associated with polyP granules in vivo [Tumlirsch et al., 2015]. PPK1a, PPK2b and PPK2c contribute to polyP synthesis in *R. eutropha*. The sizes of the proteins are enlarged relative to the polyP granule for better visibility.

vealed by fusion analysis with the enhanced yellow fluorescent protein (eYFP). PPK1b and PPK2b are localized near one of the cell poles and form fluorescent foci (apart from polyP granules) when fused to eYFP, whereas eYFP-PPK2a is soluble in the cytoplasm [Tumlirsch et al., 2015]. Only one of the seven PPKs of *R. eutropha*, PPK2c, has been biochemically characterized so far [Hildenbrand et al., 2019; Hildenbrand et al., 2020] and showed a very broad nucleotide specificity. The main function of PPK2c presumably is to replenish GTP and other NTP pools during times of enhanced demand on the expense of previously accumulated polyP. The properties of the other PPKs in *R. eutropha* are currently determined in our laboratory. Several proteins are associated with polyP granules in vivo in addition to the four PPKs (Fig. 1). These are PptA, PptB, PPI18 and PPI27: PptA and PptB both have a so-called conserved histidine alpha-helical domain (CHAD) [Iyer and Aravind 2002; Tumlirsch and Jendrossek 2017]. Expression of fusions of *pptA* or *pptB* with

eyfp and staining of the cells with 4',6-diamidin-2-phenylindole (DAPI) resulted in cell-pole localized polyP granules with attached eYFP-PptA or eYFP-PptB in contrast to polyP granules residing in the middle region of the cell as usual. The same result was obtained when fusions of *eyfp* with CHAD-encoding genes of other proteobacteria were expressed in *R. eutropha*. Since eYFP-CHAD proteins were soluble in a polyP-deficient background (*R. eutropha* in which all seven *ppk* genes had been deleted), these results indicate that CHAD proteins specifically bind to polyP surfaces and can direct the polyP granules to the cell poles if they are overexpressed (Ppt stands for polyP targeting). The designation of polyP-associated CHAD-motif containing proteins as “phosins” in analogy to phasins, that are polyhydroxyalkanoate (PHA) associated proteins without obvious catalytic function, has been suggested for PptA and PptB [Tumlirsch and Jendrosseck 2017]. Meanwhile, phosins were identified independently in other species and their binding specificity to polyP has been demonstrated [Lorenzo-Orts et al., 2019; Werten et al., 2019]. The functions of the two remaining polyP associated proteins in *R. eutropha* (PPI18 and PPI27; PPI stands for polyP interacting protein) is unknown. Both proteins co-localize with polyP granules (as fusion with eYFP) in some but not in all growth stages [Tumlirsch 2017].

The unusual high number of seven *ppk* genes in the genome of *R. eutropha* suggests that polyP has multiple, presumably more functions than only that of a storage material for phosphorus. We thought that the exceptionally high number of *ppk* genes predestinates *R. eutropha* as an ideal species to study the different functions of polyP by knocking out *ppk* genes and to study the phenotypic effects, and this was the basis of the present study. *E. coli* was included for comparison.

Results and Discussion

Formation of PolyP in *R. eutropha* and *E. coli*

PolyP granules can be visualized by staining cells with DAPI and imaging at a DAPI-polyP-specific wavelength of ≈ 515 nm (in comparison to DAPI-DNA at ≈ 465 nm) [Klauth et al., 2006]. *R. eutropha* forms one or two DAPI-stainable discrete polyP granules (Fig. 2 of Tumlirsch and Jendrosseck [2017]) when the cells are grown under optimal supply with nutrients (such as nutrient broth medium) and appropriate physical parameters (moderate temperature [30°C] and pH ≈ 7). PolyP granules in *R. eutropha* cells are located in the central (nucleoid) region. In

contrast, *E. coli* is known to synthesize polyP only under stress conditions such as nutrient downshift, elevated temperature or oxidative stress [Rao et al., 1998; Kornberg et al., 1999; Gray and Jakob 2015] but the formation of polyP granules in non-stressed *E. coli* wild-type strains has not been demonstrated so far. To determine how polyP formation in *R. eutropha* is influenced by stress conditions, we analyzed the formation of polyP before and after exposure to different stress conditions. For most experiments, *E. coli* was used as a control. The experiments were performed with both wild-type (WT) strains and the corresponding polyP-free mutant strains (*R. eutropha* Δppk -all in which all seven *ppk* genes had been deleted and *E. coli* Δppk ; *E. coli* has only one *ppk* gene [Akiyama et al., 1992]).

Heat Shock and Growth at Elevated Temperatures Have No Effect on PolyP Formation in *R. eutropha*

R. eutropha and *E. coli* (WT and Δppk mutants) were grown on NB or LB medium at 30 and 37°C, respectively, and the polyP contents were determined by fluorescence microscopy and polyP extraction from lyophilized samples. PolyP determination was repeated after applying a short-time (0–20 min) heat stress (55°C). Figure 2a shows *E. coli* and *R. eutropha* cells stained with DAPI before and after a heat stress impulse. Discrete DAPI-polyP foci were not detected in *E. coli* cells under any condition. Occasionally, *E. coli* cells with diffuse DAPI signals in the cell periphery or near the cell poles were observed. When the cells were imaged in brightfield, heat-stressed cells often revealed dark bubble-like signals near the poles and/or the cell periphery. Some of these signals seemed to co-localize with the DAPI signal. However, the DAPI and the bright field signals were detected not only in the WT but were also found in the Δppk mutant to the same extent. Therefore, these signals are unlikely to indicate the presence of polyP. They might be a response of the cells to the heat stress and could represent aggregates of heat-dam-

Fig. 2. a Formation of polyP in *E. coli* and in *R. eutropha*. *E. coli* WT and *E. coli* Δppk (upper left) and *R. eutropha* WT and *R. eutropha* Δppk -all (upper right) were grown in LB or NB medium and exposed to a 20 min or 10 min heat shock at 55°C, respectively, as described in the Method section. Samples taken before and after the heat shock were stained with DAPI and imaged in bright field and in the DAPI-polyP channel. Arrows point to DAPI-stained polyP granules. **b** PolyP formation in *E. coli* WT and *E. coli* Δppk (lower left) and *R. eutropha* WT and *R. eutropha* Δppk -all (lower right) upon incubation in MOPS-medium and exposure to bleach. Cells in inset squares were added to the images to show at least two individual cells per condition. Microscopic pictures show representative cells of two independent experiments.

(For figure see next page.)

Table 1. PolyP levels in *E. coli* and in *R. eutropha*

Strain	Medium	polyP before heat shock [$\mu\text{mol P}_i/\text{g cdw}$]	polyP after heat shock [$\mu\text{mol P}_i/\text{g cdw}$]
<i>R. eutropha</i> WT	NB	60 ± 3.3	67 ± 2.4
<i>R. eutropha</i> WT	0.9% NaCl	60 ± 3.3	65 ± 0.7
<i>R. eutropha</i> $\Delta ppk\text{-all}$	0.9% NaCl	<0.3	<0.3
<i>E. coli</i> WT	LB	<0.3	1.0 ± 0.4
<i>E. coli</i> WT	0.9% NaCl	<0.3	<0.3
<i>E. coli</i> Δppk	0.9% NaCl	<0.3	<0.3

R. eutropha: One biological replicate with three technical replicates, *E. coli* in 0.9% NaCl at least two biological replicates, in LB, one biological replicate, with three technical replicates.

Fig. 3. Growth of *R. eutropha* WT and $\Delta ppk\text{-all}$ on NB medium. In a microtiter plate, 200 μL of NB medium was inoculated with an 18 h NB seed culture of *R. eutropha* WT (black circles) and $\Delta ppk\text{-all}$ (open circles), respectively, in six technical replicates to an initial OD_{600} of about 0.05. Cells were cultivated at the indicated temperatures for 15 h with constant orbital shaking. The graphs display mean $\pm$ SD of six technical replicates.

aged cell components. In contrast, *R. eutropha* WT cells formed one or two distinct, globular-shaped granule-like structures that were clearly detectable in the DAPI-polyP channel. This was independent of whether a heat stress impulse was applied to the cells or not. Globular, DAPI-stainable structures were not observed in the *R. eutropha* $\Delta ppk\text{-all}$ mutant under any condition being in agreement with the polyP-negative phenotype of this mutant in our previous work [Tumlirsch and Jendrossek 2017]. In summary, our data indicate that microscopically detectable polyP granules are not formed in *E. coli* either in heat-stressed or in non-stressed cells but that polyP granules

in *R. eutropha* WT are formed independent of the application of a heat-stress impulse.

Next, we directly determined the amount of formed polyP in both species by polyP extraction and subsequent colorimetric phosphate quantification. Non-stressed *E. coli* cells (WT and the Δppk mutant) did not contain significant amounts of polyP (<0.3 $\mu\text{mol P}_i/\text{g}$ of cell dry weight [cdw]) (Table 1). Very low amounts of polyP of $1.0 \pm 0.4 \mu\text{mol P}_i/\text{g cdw}$ were detected in heat-stressed *E. coli* WT cells, but no significant polyP content was detected in the Δppk mutant after heat stress. Apparently, *E. coli* is able to synthesize trace amounts of polyP after a heat-stress impulse, but this amount is too low to be reliably detected by fluorescence microscopy. In contrast, much higher levels of $\approx 60 \mu\text{mol P}_i/\text{g cdw}$ were determined for *R. eutropha* WT that did only marginally increase to 65 (in NaCl) or 67 (in NB medium) $\mu\text{mol P}_i/\text{g cdw}$ upon heat stress. In an independent repetition of this experiment, we even determined a slight decrease in the polyP content after heat stress (not shown). From these data, we conclude that the polyP levels in *R. eutropha* are generally much higher than in *E. coli* but that heat stress does not influence the polyP levels to a large extent in the two species. As expected, no polyP was detected in the $\Delta ppk\text{-all}$ mutant under any condition and confirmed the polyP-negative phenotype of the mutant strain.

PolyP Does Not Increase Survival of *R. eutropha* after Heat Stress

To determine, whether polyP could have an impact on survival of the bacteria after exposure to a heat stress impulse, we determined survival of WT cells and of the polyP-free $\Delta ppk\text{-all}$ mutant cells after the heat stress (0–20 min 55°C). However, we could not find a significant difference in the survival of the WT versus the polyP-free

Fig. 4. Effect of bleach (HOCl) on survival of *R. eutropha* and *E. coli*. *R. eutropha* WT and Δppk -all (a) and *E. coli* WT and the Δppk mutant (b) were cultivated in MOPS-medium with 1.32 mM K_2HPO_4 and 0.2% fructose (or glucose in case of *E. coli*) at 30°C or 37°C and orbital shaking. After 6 h, 10 mL cell suspension was transferred to fresh flasks and treated with PBS (control) or with 2 mM HOCl. Samples were taken after 15 min, diluted in PBS and stained with propidium iodide. Three biologic replicates with $n = 10,000$ events were measured by flow cytometry (a) or 2 biological replicates with $292 \leq n \leq 606$ cells were counted by fluorescence microscopy (b). Error bars show standard deviation.

mutant (suppl. Fig. s1). When the survival of *E. coli* cells after exposure to a heat stress impulse was tested analogously, *E. coli* WT and the Δppk mutant revealed a similar sensitivity (suppl. Fig. s2). Only if highly diluted cell suspensions of OD = 0.002 were heat-challenged, *E. coli* WT cells exhibited higher viability compared to the Δppk mutant. We do not know why the Δppk mutant was more heat sensitive at diluted cell concentrations versus high cell concentrations.

PolyP in R. eutropha Does Not Influence Growth at High Temperatures

The polyP levels in *R. eutropha* did not change dramatically after application of a short time heat impulse as shown above. To evaluate whether polyP could be advantageous for growth at temperatures near the grtemperatura maximum we compared growth of *R. eutropha* WT and the polyP-free Δppk -all mutant at different temperatures. As evident from Figure 3, no difference in growth was determined between *R. eutropha* WT and the polyP-free Δppk -all mutant. Both strains showed good growth at 30–42°C but could not multiply at 45 or 47°C. At 42°C, growth of both strains was slightly reduced compared to 37 or 30°C suggesting that 42°C is near the upper temperature limit of *R. eutropha*. However, the WT strain and the Δppk -all mutant showed the same growth characteristics at all tested temperatures. We conclude that polyP has no function in heat tolerance in *R. eutropha* and does not allow a better growth at high temperatures.

PolyP Does Not Increase Oxidative Stress Resistance

Many reports have previously described the beneficial effects of polyP for resistance of *E. coli* and other species

against oxidative stress such as hydrogen peroxide or bleach [Casey et al., 2013; Dahl et al., 2017; Groitl et al., 2017; Sultana et al., 2020]. To test if oxidative stress has an impact on survival of *R. eutropha*, we exposed the cells with 2 mM bleach (HOCl). *E. coli* cells were also tested for comparison. To this end, the WT and polyP-free mutant strains of both species were incubated in MOPS-buffered medium supplemented with 1.3 mM phosphate and 0.2% fructose (*R. eutropha*) or 0.2% glucose (*E. coli*). After incubation for 3 h, the cells were challenged with 2 mM bleach for 15 min. Cell survival was estimated by spotting appropriate dilutions on agar media and was additionally monitored by the fraction of cells that were resistant to the uptake of propidium iodide (PI). Although the number of PI-positive *R. eutropha* cells increased upon exposure to bleach, we could not detect a significant difference between WT and Δppk -all cells (Fig. 4). This was in agreement with a comparable number of viable cells between the WT and the Δppk -all mutant in the agar-spot assay (not shown). For *E. coli*, however, we determined a significant difference between the WT and the Δppk -strain in sensitivity toward exposure to bleach: HOCl-treated cultures of *E. coli* Δppk displayed a large increase in PI-positive Δppk cells to ~34%, whereas the WT showed only an increase from 5 to 9% PI-positive cells. The difference in survival between the WT and the Δppk mutant was confirmed by the agar-spot-assay (not shown).

Exposure to Bleach Does Not Affect the Formation of PolyP Granules

To test whether exposure to bleach stimulates the formation of polyP, we used the cells of the experiment described above to follow the formation of polyP granules

Fig. 5. Effect of bleach on the polyP levels in *R. eutropha* and *E. coli*. *R. eutropha* WT and Δppk -all (a) and *E. coli* WT and the Δppk mutant (b) were incubated for 3 h in a MOPS-medium with 1.32 mM K_2HPO_4 and 0.2% fructose (or 0.2% glucose in case of *E. coli*) at 30°C and 37°C respectively. At $t = 0$ 50 mL cell suspensions of each strain were transferred to fresh flasks and treated with PBS (control) or with 1 mM HOCl. Samples were taken after 60 min.

fluorescence microscopically and to quantify the amount of extractable polyP in cell samples. As shown in Figure 2b, most *E. coli* WT and *R. eutropha* WT cells formed one (rarely two) DAPI-stainable polyP granules during incubation in the MOPS-phosphate-sugar medium. Interestingly, the number of DAPI-polyP granules did not further increase upon exposure of *E. coli* or *R. eutropha* WT cells to bleach. As expected, the *R. eutropha* Δppk -all and Δppk *E. coli* mutant strains did not form DAPI-stainable polyP granules under any condition. When the amount of extractable polyP was determined, *R. eutropha* WT revealed a polyP content of 45 $\mu\text{mol P}_i/\text{g cdw}$ that remained almost constant upon exposure to bleach (Fig. 5a). When *E. coli* WT was tested, a high polyP content of 41 $\mu\text{mol P}_i/\text{g cdw}$ was determined in MOPS-phosphate-glucose medium that increased to 67 $\mu\text{mol P}_i/\text{g cdw}$ after exposure to bleach (Fig. 5b). The formation of microscopically detectable polyP granules and the determination of a high value of 41 $\mu\text{mol P}_i/\text{g cdw}$ before treatment of *E. coli* WT with bleach was unexpected as no polyP was detected in cells after growth in LB medium (Fig. 2a). We assume that incubation of *E. coli* cells in a phosphate- and carbon source-containing MOPS-medium that lacks other nutrients enables the cells to produce more ATP by respiration of glucose than can be consumed by metabolism. The surplus of ATP is transiently accumulated in the form of polyP granules. Presumably, the nutrient downshift from complex medium to a MOPS-phosphate-glucose solution provoked stress to the cells that lead already to Dsk-dependent [Gray 2019] and ppGpp-associated polyP formation (and/or inhibition of polyP degradation by exopolyphosphatase) so that the addition of bleach at a later time-point only marginally further increased the forma-

tion polyP. These findings suggest that polyP has the function of a barrage for ATP during periods of a transient misbalance of ATP-producing and ATP-consuming reactions.

PolyP Has No Impact on the Motility of R. eutropha

PolyP has an impact on motility of several bacterial species such as *P. aeruginosa*, *Pseudomonas syringae*, *Bacillus cereus* or *Myxococcus xanthus* [Rashid et al., 2000; Shi et al., 2004; Zhang et al., 2005; Fraley et al., 2007; Hosain et al., 2008]. To analyze if this is also true for *R. eutropha*, we determined motility of *R. eutropha* WT and the Δppk -all mutant in swimming and swarming assays. However, we could not find any difference between the WT and the Δppk -all mutant (suppl. Fig. s3). We conclude that polyP has no impact on motility in *R. eutropha*.

Growth and Formation of PolyP in Mineral Salts Medium with or without Phosphate.

Surprisingly, the presence of polyP in *R. eutropha* WT had no detectable advantage for survival in our experiments, and we could not detect a phenotype of the Δppk -all mutant under any of the tested conditions shown above (heat shock/high temperature, reactive oxygen species, motility). Apparently, polyP has no obvious function in coping with these stressors. The findings suggest that polyP must have another function in *R. eutropha*. The most obvious one is the function as a reservoir for phosphorus. *R. eutropha* was originally isolated from the spring of the Wende river near Göttingen/Germany [Wilde 1962]. The concentrations of nutrients such as nitrogen or phosphorus sources are usually low in aquatic ecosystems and are even lower in the springs of most riv-

Fig. 6. Growth of *R. eutropha* WT and $\Delta ppk\text{-all}$ on Tris-buffered mineral salts medium with 0.8% fructose and 0.02% KH_2PO_4 as only carbon and phosphorus sources. Both cultures were inoculated with washed cells obtained after two subsequent NB cultures (30°C, over night and then for 24 h). **a** Optical density at 600 nm (OD_{600}). **b** PolyP content by means of phosphate residues (P_i) per

g of cellular dry weight (cdw) was determined by exopolyphosphatase-digestion of isolated polyP and colorimetric measurement with an antimony-tartrate-ascorbate assay. **c** PHB content per g cdw was determined by gas chromatography after acidic methanolysis.

ers. Therefore, it makes sense for *R. eutropha* to store an excess of phosphorus intracellularly in the form of polyP granules, and this might be one explanation why polyP is regularly found in stationary cells of *R. eutropha* cultures that are not limited by phosphorus. To determine whether the presence of polyP has an advantage for *R. eutropha* under conditions of limited nutrient supply, we tested growth, polyP content, cell numbers and cell viability in a mineral salts medium (MSM) with restricted supply of phosphorus. To this end, we cultivated *R. eutropha* WT and the $\Delta ppk\text{-all}$ mutant in a modified MSM medium

with 0.8% fructose in which phosphate, which is usually present in high concentration as a buffer, was replaced by tris(hydroxymethyl)aminomethane (Tris). Phosphate was added at 0.02% (1.3 mM) (Fig. 6) or was completely absent (Fig. 7). At selected time points, samples were taken and analyzed for optical density (OD), polyP and PHB content. *R. eutropha* WT and the $\Delta ppk\text{-all}$ mutant grew comparably well on Tris-MSM in the presence of fructose and 0.02% phosphate and reached OD_{600} values of ≈ 5 after 2 days (Fig. 6a). Only in the exponential growth phase between 12 and 36 h, the OD_{600} values of the $\Delta ppk\text{-all}$

Fig. 7. Growth of *R. eutropha* WT and $\Delta ppk\text{-all}$ on Tris-buffered mineral salts medium with 0.8% fructose but without a phosphate source. Both cultures were inoculated with washed cells obtained after two subsequent NB cultures (30°C, overnight and then for 24 h). **a** Optical density at 600 nm (OD₆₀₀). The black arrow indi-

cates the addition of 0.02% KH₂PO₄ after 144 h of cultivation without phosphate. **b** PolyP content by means of phosphate residues (P_i) per g of cellular dry weight (cdw). **c** PHB content was analyzed by gas chromatography after acidic methanolysis of two biological replicates. **d** Cell density at 72 h.

mutant were slightly lower than those of the WT but caught up with WT levels after ≥ 48 h. PolyP was detectable only in the WT. The polyP levels were relatively high at the beginning of growth (80 μmol P_i/g cdw) but then decreased to levels below 5 μmol P_i/g cdw (Fig. 6b). No polyP was detected in the $\Delta ppk\text{-all}$ mutant and confirmed the inability of this mutant to produce detectable amounts of polyP. Both strains accumulated large amounts of PHB up to 70% PHB of cellular dry weight (cdw) (Fig. 6c). However, PHB contents of the $\Delta ppk\text{-all}$ mutant during growth were slightly lower than in the WT, and this could explain the lower OD values of the mutant versus WT. The determination of high levels of PHB indicated that the cells were not limited by the carbon source (0.8% fructose) but were limited by phosphorus (0.02%), and this

explains why the amount of polyP decreased during growth of the WT. The high values for polyP at the beginning of the experiment are due to polyP formation from the pre-culture.

If polyP has the function of a P-reservoir, *R. eutropha* WT should have an advantage over a polyP-deficient mutant in media with growth-limiting amounts of phosphorus. To test this assumption, *R. eutropha* was first grown in NB medium; under these conditions, the cells form polyP at high levels. These polyP-rich cells were then transferred to a fresh Tris-MSM with fructose but without any P-source. As shown in Figure 7a, *R. eutropha* WT showed reduced but still substantial growth compared to the culture with phosphate in the first 24 h. However, growth slowed down continuously after 12 h reaching a

final optical density of OD₆₀₀ of ≈ 1 after 144 h. In contrast, the $\Delta ppk\text{-}all$ mutant grew slower than the WT in the absence of phosphate and reached an OD₆₀₀ value of only ≈ 0.6 . The optical density of bacterial cultures can be also influenced by the formation of PHB. Since the high carbon concentration (fructose) in the absence of a phosphate source should support strong PHB accumulation, we also determined the PHB content of the cultures. As expected, high amounts of PHB up to 60% PHB of cdw were determined. Interestingly, the PHB contents of WT cells were about 10% higher (reaching a maximum of 60% after 72 h) than those of the $\Delta ppk\text{-}all$ mutant which reached only 48.5% at 72 h. Therefore, a substantial amount of the higher OD₆₀₀ values of the WT compared to the $\Delta ppk\text{-}all$ mutant presumably is caused by the higher PHB content of the WT. To investigate if the presence of polyP really enables better growth, we determined the total number of cells of the WT and the $\Delta ppk\text{-}all$ culture at 72 h. As evident from Figure 7d, the cell numbers of the WT culture were substantially higher ($1.33 \times 10^8 \pm 2.88 \times 10^6$) compared to the $\Delta ppk\text{-}all$ culture ($1.02 \times 10^8 \pm 3.75 \times 10^5$ [77% of WT level]), while 88% of the WT cells ($n = 540$ cells) and 89% of the $\Delta ppk\text{-}all$ mutant cells ($n = 359$ cells) were still viable as revealed by PI staining (not shown). The mean cell size (measured as cell length) was also similar for the WT and the $\Delta ppk\text{-}all$ mutant (1.7–1.8 μm) but was lower compared to the beginning of the experiment (≈ 2.4 μm , 1.7–3.4 μm). These data lead to the conclusion that both the higher number of cells in the WT culture as well as the stronger accumulation of PHB contribute to the higher OD₆₀₀ values of the WT compared to the $\Delta ppk\text{-}all$ mutant and also confirmed that the presence of polyP leads to a substantial growth advantage of the WT over the polyP-deficient mutant.

To verify whether the polyP material was consumed by the WT during growth, we determined the levels of polyP at selected time points fluorescence microscopically and by polyP quantification after extraction from the cells (Fig. 7b). *R. eutropha* WT cells were rod-shaped, and most of the cells harbored one prominent polyP granule at the beginning of the growth experiment in the phosphate-free medium. A similar result was observed after 6 h of growth. After 12 h and at later time points the number of cells with DAPI-stainable polyP granules decreased and the cells became shorter and shorter reaching an almost coccoid morphology at the end of the experiment (suppl. Fig. s4a). In accordance with fluorescence microscopical analysis, *R. eutropha* WT revealed polyP levels of ≈ 60 $\mu\text{mol P}_i/\text{g cdw}$ at the beginning of the experiment, and these values constantly decreased to sub-detectable

levels within 36 h. In contrast, polyP was not detected in the $\Delta ppk\text{-}all$ mutant at any time point either in the form of DAPI-stainable granules or in the form of extractable polyP (suppl. Fig. s4b). When 0.02% phosphate was added to the cultures after 144 h, growth of the WT and of the $\Delta ppk\text{-}all$ mutant immediately resumed and reached OD₆₀₀ values of ≥ 7 (Fig. 7a) thereby confirming that the absence of phosphate was the growth-limiting factor in the experiment. Large amounts of PHB were accumulated by both strains. Remarkably, the amount of PHB was substantially higher in the WT compared to the $\Delta ppk\text{-}all$ mutant at all time points (Fig. 7c).

In summary, our data showed that the formed polyP in the WT was re-used as a phosphate source and allowed poor growth and for some (unknown) reason supported PHB accumulation to a 10–15% higher extent than in the $\Delta ppk\text{-}all$ mutant. However, the growth advantage of polyP under phosphate starvation in the WT seems to be only marginal. If the main function of polyP would be that of a P-reservoir, why do the cells form only one tiny polyP granule (in the stationary phase of NB-grown cultures) and not several large (voluminous) polyP granules as this is, for example, the case for PHB granules in PHB-accumulating cells? Formation of large polyP granules in *R. eutropha* is possible and has been demonstrated for *R. eutropha* previously by constitutive overexpression of *ppk2c* [Hildenbrand et al., 2020]. However, a simultaneous accumulation of high amounts of PHB AND high amounts of polyP leads to growth defects and inhibition of cell division resulting in the formation of unusually long cells [Tumlirsch et al., 2015; Hildenbrand et al., 2019].

Materials and Methods

Growth Media and Growth Conditions

R. eutropha and *E. coli* strains (Table 2) were grown in nutrient broth (NB, 0.8%) and in LB medium (10 g/L Trypton, 5 g/L yeast extract, 10 g/L NaCl) at 30 or 37°C, respectively. For most experiments with *R. eutropha*, the cells were grown overnight in NB medium (20 mL in a 100 mL Erlenmeyer flask) under continuous shaking at 30°C. 0.1 volume of this first seed culture was used to inoculate a second seed culture on the same medium (20–100 mL in an appropriately dimensioned flask) and was grown for 24 h at 30°C. Cells of this culture were used either directly or were harvested by centrifugation, washed with and resuspended in 0.9% NaCl (heat shock experiments) or in phosphate-free tris(hydroxymethyl)amino-methane (Tris)-MSM (experiments $\pm$ phosphate), respectively (see below). Growth experiments with *R. eutropha* strains at elevated temperatures were conducted in microtiter plates with 250 μL wells containing 200 μL NB medium. Wells were inoculated with cells from an NB overnight seed culture to an optical density (OD₆₀₀) of 0.05. Growth was monitored by OD₆₀₀ determination at temperatures as indicated with orbital shaking in a microplate reader

Table 2. Bacterial strains used in this study

Strain	Relevant characteristic	Source/reference
<i>Escherichia coli</i> LJ110 (W3110)	<i>E. coli</i> K-12 derivative, F ⁻ λ ⁻ rph-1 INV (<i>rrnD</i> , <i>rrnE</i>) W3110 Fnr ⁺	Bachmann, 1972
<i>E. coli</i> LJ110 Δ <i>ppk</i>	<i>E. coli</i> LJ110 with chromosomal deletion of <i>ppk</i>	Tumlirsch et al., 2015
<i>Ralstonia eutropha</i> H16	<i>R. eutropha</i> wild-type strain	DSMZ 428
<i>R. eutropha</i> H16 Δ <i>ppk-all</i>	Chromosomal deletion of seven <i>ppk</i> genes: <i>ppk1a</i> , <i>ppk1b</i> , <i>ppk2a</i> , <i>ppk2b</i> , <i>ppk2c</i> , <i>ppk2d</i> , <i>ppk2e</i>	Tumlirsch and Jendrossek, 2017

(BioTek, Bad Friedrichshall, Germany). Alternatively, *R. eutropha* was cultured in MSM with carbon source as indicated. A Tris-buffered MSM of the following composition (values per liter) with or without the presence of 0.02% KH₂PO₄ was used: 6.06 g Tris, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH₄Cl, 0.2 g MgSO₄ × 7H₂O, 0.01 g CaCl₂ × 2H₂O, 0.005 g ferri-ammonium-citrate (C₆H₈O₇ × nFe × nNH₃), 1:10,000 (v/v) trace element solution SL6 (per 1L: 1 g ZnSO₄ × 7H₂O, 0.3 g, MnCl₂ × 4H₂O, 3 g H₃BO₃, 2 g CoCl₂ × 6H₂O, 0.1 g CuCl₂ × 2H₂O, 0.2 g NiCl₂ × 6H₂O, 0.3 g Na₂MoO₄ × 2H₂O), pH 7.4. Fructose was added separately after sterilization to 0.8%.

Oxidative Stress Tests

NB-grown *R. eutropha* cells were incubated in a morpholino-propane sulfonic acid (MOPS) MSM with 4.18 g/L MOPS, 0.23 g/L K₂HPO₄ (pH 7.1) and 0.2% fructose (0.05 volumes of NB culture as seed culture) for 6 h at 30°C. 10 mL portions were transferred to fresh flasks and challenged with 100 μL phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.42 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄) (control) or with 2 mM NaOCl (in 100 μL PBS). After 15 min of treatment with bleach, the samples were centrifuged, the supernatant was removed and the cells were resuspended in 20 mM MOPS buffer containing 20 mM Na₂S₂O₃. Subsequently, the cells were diluted 1:50 with PBS. Cells were stained with 2 μg PI/mL H₂O for 15 min in the dark and analyzed by flow cytometry using a Cytex NL-2000 (excitation 530 nm, emission 615 nm) or by fluorescence microscopy. *E. coli* cells were treated analogously with the exception that fructose was replaced by glucose.

For polyP quantification and viability tests, 200 mL MOPS MSM with 1.32 mM phosphate and 0.2% fructose or glucose were inoculated with an NB or LB overnight pre-culture of *R. eutropha* and *E. coli* to an OD₆₀₀ = 0.3, cultivated at 30°C or 37°C, respectively, for 3 h with continuous shaking. 50 mL portions were transferred to fresh flasks and treated with H₂O as control or with 1 mM NaOCl (500 μL of 100 mM NaOCl stock in H₂O). After incubation for 1 h under cultivating conditions, 1 mL portions were centrifuged, 800 μL supernatant was removed and replaced by 800 μL of 20 mM Na₂S₂O₃ in 20 mM MOPS to stop the reactions. For viability test, cells were serially diluted from 10⁰ to 10⁻⁴ in 0.9% NaCl (4°C pre-cooled for *E. coli*, room temperature (RT) for *R. eutropha*). 2 μL of each dilution was spotted on an LB or NB agar plate and incubated at 37°C (*E. coli*) or at 30°C (*R. eutropha*), respectively.

Cells (10 μL) were stained with DAPI and PI for fluorescence microscopy (for details, see below). The remaining 49 mL sample was centrifuged at 5,087 g, resuspended in 20 mL 20 mM Na₂S₂O₃

in 20 mM MOPS and washed with 0.9% saline. Cells were lyophilized for polyP extraction and quantification.

Heat Shock Experiments

NB (*R. eutropha*) or LB (*E. coli*) cultures were transferred to fresh medium and grown for 24 h at 30 and 37°C, respectively. Cells were harvested by centrifugation in an Eppendorf centrifuge (1 min, 5,087 g at RT) and resuspended in 0.9% NaCl. Cell density was adjusted to an optical density (OD₆₀₀) of 0.2 or 20. 10 μL of cells were added to 990 μL of pre-heated (55°C) 0.9% NaCl in a 2 mL microfuge tube so that the resulting OD₆₀₀ was 0.002 or 0.2. Cells were incubated at 55°C for 0–20 min with constant shaking (450 rpm in a thermo shaker, Biometra, Germany). 100 μL samples were taken in time intervals and serially diluted from 10⁰ to 10⁻⁴ in 0.9% NaCl (4°C pre-cooled for *E. coli*, RT for *R. eutropha*). 2 μL of each dilution was spotted on an NB or LB agar plate and incubated at 30°C for 2 days (*R. eutropha*) or 37°C for 1 day (*E. coli*), respectively. For determination of the polyP content after a heat shock, the experiment was scaled up to 50 mL, and the initial cell density was raised to OD₆₀₀ of 20. The cell suspension was diluted to a final OD₆₀₀ = 0.2 in 50 mL pre-heated (55°C) 0.9% NaCl, NB or LB in a glass flask with shaking. After 10 min (*R. eutropha*) or 20 min (*E. coli*), 50 mL stressed culture was centrifuged at 5,087 g for 8 min at 4°C. The supernatant was removed, cells were washed with H₂O, and the cell pellet was lyophilized and stored until polyP extraction.

PolyP Extraction and Quantification

PolyP was extracted from lyophilized cells by neutral phenol-chloroform extraction and subsequent precipitation in ethanol as described in [Bru et al., 2016; Frank and Jendrossek 2020]. Subsequently, polyP was digested with exo-polyphosphatase and inorganic pyro-phosphatase, and released phosphate was quantified using an Sb-tartrate-ascorbate colorimetric assay as described in [Christ et al., 2019].

Motility Assays

Petri dishes were filled with 25 mL of heat-sterilized swimming or swarming agar (10 g/L Tryptone, 5 g/L NaCl, 0.3% standard agarose (Roth) for swimming assays and 8 g/L nutrient broth, 0.5% Bacto agar (Becton Dickinson, Sparks, USA) for swarming assays), and were allowed to solidify at RT for ≈1 h (swimming) or 3 h (swarming). Suspension of *R. eutropha* strains had been grown in 3 subsequent NB cultures each for 24 h at 30°C. OD₆₀₀, was adjusted to OD₆₀₀ of 0.1 with NB, and 5 μL of this suspension was spotted in five technical replicates in the center of a swimming agar

plate. 1 µL of cell suspension was spotted on swarming plates. The plates were incubated at 30°C for 1 day on a horizontal under-ground without further movement of the plates.

Determination of Cell Numbers

The determination of cell density (total cell numbers) was performed with Neubauer counting chambers (DHC-N01, Digital Bio, Suwon, Korea) according to the manufacturer's instruction.

Determination of PHB Content

PHB determination of lyophilized cell samples was performed by gas chromatography after acidic methanolysis as described in detail previously [Juengert et al., 2018].

Fluorescence Microscopy

Microscopy was carried out with a Nikon Ti-E fluorescence microscope (model number MEA53100) using a DAPI-polyP specific filter set (excitation, 387/11 nm; emission, 447/60 nm) or a long-pass filter (excitation at 562/40 nm and emission at 594 nm) for PI-stained cells. Formation of polyP granules was followed fluorescence microscopically after staining the cells with DAPI, final concentration 5 µg/mL (1:20 (v/v) 0.1 mg/mL stock solution) for 10–15 min. Live/dead staining of cells was carried out with 100 µg PI/mL (1:10 (v/v) 0.1 mg/mL stock) for 10 min. Cells were immobilized on agarose pads, and PI-positive and negative cells were counted from microscopic images.

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Statement of Ethics

Ethical approval is not required for this study (no experiments with humans or animals).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

H.R. and L.K. performed the experiments, prepared the figures and wrote parts of the manuscript. S.O. hosted HR during a 2-months stay at Brno University and helped H.R. in some experiments at Brno. D.J. designed the study and wrote most parts of the manuscript. All authors evaluated the data and read the manuscript.

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