Polyphosphate Accumulation by *Rhodobacter sphaeroides* Grown under Different Environmental Conditions with Special Emphasis on the Effect of External Phosphate Concentrations

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**Abstract:** *Rhodobacter sphaeroides* NR3 produced significant amounts of inorganic polyphosphates (polyPs) with visible volutin granules when grown phototrophically at external orthophosphate (P<sub>i</sub>) concentrations of 10 mM and above with 20 mM malate as the sole carbon source. The maximum level of the accumulated polyPs, amounting to 6.2% of the cell dry weight, was found with 300 mM P<sub>i</sub>. On the other hand, the stimulatory effect of excess P<sub>i</sub> on polyP accumulation was not so pronounced in the cells grown under aerobic-dark conditions. The fractionation of the cellular phosphorous compounds revealed that the alkali-soluble and cold acid-soluble polyPs were the major polyPs in the high P<sub>i</sub>-loaded phototrophic cultures. The phosphorus/carbon ratio in cell growth media was also found as an important factor affecting polyP accumulation in the bacterium. There was an antagonistic relationship between the polyP content and the RNA/DNA ratio during batch phototrophic growth. Ecophysiological implications of polyP accumulation in the phototrophic bacterium were also discussed.

**Key words:** polyphosphate, *Rhodobacter sphaeroides*, phototrophic bacteria

A wide variety of species of microorganisms, including bacteria, yeasts, fungi, and microalgae, are capable of inorganic polyphosphate (polyP) accumulation under certain conditions. This characteristic has attracted much interest in two ways. First, the widespread occurrence of polyPs in microorganisms, together with the fact that the polyP content of microbial cells is far greater than the cellular phosphate requirement, may suggest that they have important physiological functions, although there has been no direct evidence for a universal role for them. Second, bacterial polyP accumulation is an important means of removing excess phosphate during biological wastewater treatment. Researchers in this field have come to general agreement that there is a direct relationship between the efficiency of phosphate removal and the capacity for polyP accumulation of the existing bacteria and that the accumulated polyPs function as an energy source for anaerobic removal of organic matter by the bacteria.

Previously, we made a preliminary study for screening phosphate-tolerant strains of phototrophic purple nonsulfur bacteria for the purpose of developing a phosphate removal process using these bacteria. One of the major observations of the previous study was that *Rhodobacter sphaeroides*...
oides was most remarkable for phosphate-tolerant growth among members of the phototrophic bacteria. This observation might be explained by assuming that R. sphaeroides is capable of accumulating phosphate as polyP when exposed to excess external orthophosphate (P_i). However, few data are available on the relationships of external P_i and bacterial polyP accumulation. The mechanism of polyP formation and degradation in the phototrophic bacteria has not been extensively studied.

We have previously shown that R. sphaeroides produces large amounts of polyP when grown under carbon or sulfur limitation. In the present study, polyP formation by the phototrophic bacterium was investigated more thoroughly under different environmental conditions with special emphasis on the effect of external P_i concentrations. The main objective of this research is to obtain fundamental data on polyP metabolism in R. sphaeroides for developing a phosphate removal process using this bacterium. The relationship between polyP and nucleic acid synthesis and environmental conditions during phototrophic growth is discussed from ecophysiological viewpoints.

Materials and Methods

**Bacterial strain and cultivation.** *Rhodobacter sphaeroides* NR3 was used throughout this study. For inoculation purposes, cells were grown microaerobically in the light in MYC medium. Main cultures (1% inoculum) were made with MAT medium, which contains 20 mM malate and 15 mM NH_4Cl as the sole carbon and nitrogen sources, respectively. In some cases, the medium containing different concentrations of malate was used (see Fig. 3). The concentration of P_i (as phosphate buffer [pH 7.2]) in the medium was usually 10 mM but changed between 0.1 and 500 mM as needed. Phototrophic cultures were grown in completely filled screw-capped test tubes or bottles under incandescent illumination (ca. 5,000 lux), whereas aerobic cultures were grown in the dark in Erlenmeyer flasks on a reciprocal shaker. Incubation was at 30°C in all cases.

**Phosphate extraction and fractionation.** Cells were harvested by centrifugation, usually from cultures at the stationary phase of growth, washed twice with 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.5), and immediately subjected to phosphate extraction. Intracellular phosphorous compounds were extracted and fractionated by a modification of the Schmidt-Thanhauser-Schneider method as described elsewhere. The extraction was also performed by the method of Langen et al. with some modifications in the following manner. Washed cells were first treated with 5% perchloric acid (PCA) (twice, 10 min each) to obtain the acid-soluble extract. Nucleotide compounds were removed from this fraction by absorption to charcoal, and the acid-labile phosphate in the supernatant was used as a measure of the cold PCA-soluble polyP. Then, the pellet was treated twice with a saturated sodium perchlorate solution containing 5% PCA for 20 min each. The extract obtained with this treatment was designated the salt-soluble polyP fraction. The residue was extract twice with 0.5 M NaOH for 30 min each to obtain the alkali-soluble polyP. All procedures noted above were performed at 0 to 4°C. The residual cellular polyP was solubilized with nucleic acids by treating with 5% PCA at 100°C for 15 min. Each fraction obtained was neutralized with KOH and HCl immediately after the extraction, and stored at -20°C until analysis.

**Chemical assays.** P_i was measured by a colorimetric method as described previously. PolyP concentration of each fraction was determined asP_i liberated after hydrolysis with 1 M HCl at 100°C for 20 min. DNA was measured colorimetrically as described by Burton. The colorimetric method with the orcinol reagent was used for measuring RNA.

**Volutin staining.** Volutin granules were stained by the method of Laybourn and observed
under a Nikon microscope.

**Results and Discussion**

Effect of external $P_i$ concentrations. *Rhodobacter sphaeroides* NR3 was grown phototrophically at various $P_i$ concentrations between 0.1 and 500 mM, and the intracellular phosphorus compounds produced at each $P_i$ level were extracted and fractionated by the modified Schmidt-Thanhauser-Schneider method. As shown in Fig. 1, the polyP content was negligible in the cells grown at $P_i$ concentrations of less than 1 mM but was increased sharply with increasing $P_i$ levels when the cells were grown with 10 mM $P_i$ and above. The maximal level of the accumulated polyPs was found in the cells grown with 300 mM $P_i$. In this case, the produced polyPs of the cold PCA-soluble and the acid-insoluble fractions accounted for 2.1 and 4.1 % (as phosphorus) of the cells, respectively, on the dry weight basis. On the
other hand, the largest amount of nucleic acids (DNA + RNA) was found in the cells grown at Pi concentrations between 1 and 10 mM, and higher levels of Pi had inhibitory effects on nucleic acid synthesis. In all cases, more than 65% of the nucleic acid content was represented by RNA (data not shown), but the concentration of RNA relative to that of DNA in the cells was markedly fluctuated, depending on the external Pi level and the growth phase (see Table 2 and Fig. 4).

Microscopic experiments with volutin staining revealed that volutin granules (i.e., polyP granules) in the cells were increasingly formed with increasing levels of external Pi (Fig. 2). This observation is in agreement with that shown in Fig. 1.

The above results show that polyP and nucleic acid synthesis in *R. sphaeroides* during phototrophic growth depends on external Pi concentrations. As previously reported, this bacterium shows the highest growth rate when grown at an external Pi level between 1 and 10 mM. This is supported by the present finding that the largest amount of nucleic acids occurred in the cells grown with this range of Pi concentrations. The results provide evidence that, in the stationary cells grown on higher Pi concentrations, there is an inverse relationship between polyP and nucleic acid synthesis.

**Effect of osmotic pressure.** Our finding that excess external Pi suppressed nucleic acid synthesis but stimulated polyP accumulation in *R. sphaeroides* raises the question whether high osmotic pressure affects the formation of those compounds. To find this effect, the test organism was grown in MAT medium (containing 10 mM Pi) supplemented with either 500 mM NaCl or KCl. As shown in Table 1, the amounts of nucleic acids formed were significantly lower in the cells grown in the presence of excess NaCl or KCl than in those grown in the absence of the added salts. However, the addition of these salts did not stimulate but rather inhibited polyP accumulation. Thus, this observation could not be explained simply by the inverse relationship between polyP and nucleic acid synthesis found in the high Pi-loaded cells.

In view of the results obtained with the cells grown on high salt concentrations, it is unlikely that polyPs have an osmotical function within the cytoplasm of the phototrophic bacterium.

**PolyP formation under different growth conditions.** *R. sphaeroides* is the facultative phototroph which can grow aerobically in the dark at full atmospheric oxygen tension, as well as in the light under anaerobic conditions. Thus, we investigated the bacterium in greater detail for polyP accumulation under both growth conditions with different external Pi levels. In these experiments, the Langen's method was used for fractionating intracellular polyPs according to the degree of polymerization. This successive fractionation gives four distinct polyPs of increasing molecular weight, i.e., cold PCA-soluble, salt-soluble, alkali-soluble, and hot PCA-soluble

<table>
<thead>
<tr>
<th>Salt added</th>
<th>PolyP Cold PCA soluble</th>
<th>Cold PCA insoluble</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.038</td>
<td>0.293</td>
<td>0.068</td>
<td>0.268</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>0.011</td>
<td>0.125</td>
<td>0.051</td>
<td>0.147</td>
</tr>
<tr>
<td>500 mM KCl</td>
<td>0.014</td>
<td>0.163</td>
<td>0.056</td>
<td>0.152</td>
</tr>
</tbody>
</table>

*Results obtained with cells at the stationary phase of growth.

*10 mM Pi (as KH₂PO₄) was present in all cases.*
Polyphosphates in Rhodobacter sphaeroides 29

Table 2. PolyP and nucleic acid contents of *Rhodobacter sphaeroides* NR3 grown under different conditions

<table>
<thead>
<tr>
<th>Growth condition and Pi level</th>
<th>PolyP</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cold PCA soluble</td>
<td>Salt soluble</td>
<td>Alkali soluble</td>
</tr>
<tr>
<td>Anaerobic, light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM Pi</td>
<td>0.043</td>
<td>0.033</td>
<td>0.178</td>
</tr>
<tr>
<td>300 mM Pi</td>
<td>0.678</td>
<td>0.177</td>
<td>0.760</td>
</tr>
<tr>
<td>Aerobic, dark</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM Pi</td>
<td>0.045</td>
<td>0.041</td>
<td>0.033</td>
</tr>
<tr>
<td>300 mM Pi</td>
<td>0.112</td>
<td>0.140</td>
<td>0.362</td>
</tr>
</tbody>
</table>

*Results obtained with cells at the stationary phase of growth.*

dolyPs.

Table 2 shows comparative data on the variation of the intracellular polyP contents as a function of cell growth conditions and external Pi concentrations (10 and 300 mM). The stimulatory effect of the excess external Pi on polyP accumulation could be found clearly under phototrophic growth conditions, as already reported above, whereas this effect was less pronounced in the cells grown aerobically in the dark. Also, there were some differences in the type of the accumulated polyPs between the cells grown under phototrophic and chemotrophic conditions with high Pi loading. The proportions of the cold PCA-soluble and hot PCA-soluble polyPs to the total polyPs were much higher in the light-grown cells than in the dark-grown ones, although the alkali-soluble polyP constituted a major part in both types of the high Pi-loaded cells. When loaded with 10 mM Pi, the phototrophically grown cells contained a twofold higher amount of the total polyPs than did the chemotrophically grown cells.

The relative proportions of different types of polyPs in cells grown with 10 mM Pi were changed depending on the growth phase (data not shown). The salt-soluble polyP was the major polyP in the phototrophic culture at the exponential phase of growth but decreased markedly during the stationary stage. In the chemotrophic culture, the alkali-soluble polyP predominated at the exponential phase but was reduced at the stationary phase. These results are similar to those of Kulaev et al. obtained with *Rhodospirillum rubrum*. The relative proportions of the four polyP fractions from the high Pi-loaded cells remained relatively unchanged at all growth stages (data not shown).

Effect of external P/C ratios. The results obtained with the phototrophic and chemotrophic cultures suggest that not only external Pi concentrations but also the availability of light are important factors affecting polyP accumulation by the phototrophic bacterium. Probably, excess supply of both Pi and light energy far greater than the cellular requirement for balanced growth induces cells to form large amounts of polyPs. If this is true, the phosphorus/carbon ratio in growth media should be considered another key factor influencing polyP accumulation under phototrophic conditions, because decreased levels of the carbon source against to the phosphate source may result in producing extra amounts of phosphate not directly needed for growth.

To confirm this, we cultured *R. sphaeroides* at three different concentrations of malate (0.2, 2, and 20 mM) as the sole carbon source and investigated the effect of external Pi concentrations on the polyP formation at each carbon level. As shown
in Fig. 3, the culturing at a given P/C ratio resulted in producing similar amounts of polyPs, irrespective of the absolute carbon and phosphorus concentrations added. A direct relationship between the polyP content and the P/C ratio was clearly found, suggesting the importance of the P/C balance in growth media for polyP formation by the phototrophic bacterium under phototrophic conditions. The above finding is consistent with our previous observation that carbon limitation triggers polyP synthesis in phototrophically grown *R. sphaeroides*.

**Relationships of polyP contents and RNA/DNA ratios.** It is common knowledge that the RNA content of cells is directly proportional to the growth rate, whereas the DNA content remained relatively unchanged. Therefore, the RNA/DNA ratio may be used as a measure of the growth rate. It has been shown that polyP formation is inhibited during rapid growth but is accelerated when nutrient imbalance or deficiency causes the growth rate to decline. In this respect, it is interesting to find whether there is the antagonistic relationship between the polyP content and the RNA/DNA ratio.

To make clear this relationship, we monitored the changes in the polyP content and the RNA/DNA ratio during batch phototrophic growth at different P₁ levels (10, 100, and 300 mM). All data on those parameters obtained with the phototrophic cultures at various growth stages were plotted, as shown in Fig. 4. It was clear that the polyP content was increased with decreasing the RNA/DNA ratio. The line drawn to show this relation was not linear but passed through two phases with a bend at a RNA/DNA ratio of about 5. Namely, the slight increase in the polyP content against the decrease in the RNA/DNA ratio was observed when the ratio was more than 5. On the other hand, when the ratio was less than 5, the polyP level was sharply increased with lowering the ratio.

![Fig. 3. PolyP content of phototrophically grown *R. sphaeroides* NR3 as a function of external P/C ratios (w/w). Cells were grown with 0.2 (■), 2 (▲), and 20 (●) mM malate as the carbon source with a range of P₁ concentrations of 0.1—3, 1—30, and 10—300 mM, respectively. Results were obtained with cells at the stationary phase of growth.](image1)

![Fig. 4. Relationship between the polyP content and the RNA/DNA ratio in phototrophically grown *R. sphaeroides* NR3. Cells were grown at a P₁ concentration of 10 (■), 100 (▲), and 300 (●) mM. Data obtained with cells at various growth stages were plotted.](image2)
The results shown in Fig. 4 suggest that the cells with a RNA/DNA ratio of less than 5 may be in a physiological state that ATP and P$_i$ as the phosphorus source are more than essential for the cellular requirement and thus are mostly or entirely converted to polyPs as the storage material. This is the case in the cells grown with 100 and 300 mM P$_i$, as the RNA/DNA ratio in those cells was less than 5 during the overall growth phases. In contrast, the ratios of more than 5 are suggestive of the cells in which ATP and P$_i$ are used mainly for maintaining metabolic processes for balanced growth.

Ecophysiological implications of polyP accumulation. We have reported above that polyP formation by phototrophically grown R. sphaeroides is positively affected by adding increasing concentrations of P$_i$. This is true when the carbon source is the limiting factor for growth. Thus, the P/C ratio, rather than the absolute P$_i$ concentration, in cell growth media is of primary importance for polyP accumulation in the phototrophic bacterium. The finding that the excess P$_i$-stimulated polyP accumulation was found mainly in phototrophic cultures indicates the necessity of light energy for this process. Since the phototrophic bacteria generate ATP, needed for polyP synthesis, through light-dependent cyclic photophosphorylation, they can accumulate polyPs in the absence of exogenous carbon energy sources, unlike chemoheterotrophic bacteria, provided that P$_i$ and light energy are available. In fact, when the test organism was incubated for 24 h in carbon-free MAT medium under both anaerobic-light and anaerobic-dark conditions, it accumulated a great amount of polyPs only in the light (data not shown). This suggests the possibility of developing a photobiorreactor with R. sphaeroides for removing phosphate from water with high P/C ratios.

The ecological significance of R. sphaeroides and other phototrophic bacteria in the phosphorus cycle in natural environments is presently unknown. However, in view of its capacity for P$_i$ uptake and polyP accumulation, it can be assumed that the phototrophic bacterium plays a role in removing phosphate in light-exposed eutrophic environments, like the oxygenic phototrophs, even if the conditions are unfavorable for their growth. In concurrent in situ analyses with electron microscopy and fluorescent microscopy, we have found that the phototrophic bacteria found in some eutrophic ponds and lakes contained sphericial electron-dense inclusions, i.e., polyP granules, and that the nucleic acid content of such cells are significantly low compared to that of rapidly growing cells (unpublished data).

The phosphate accumulation by the excess P$_i$-loaded cells of R. sphaeroides we reported here can be considered a kind of “luxury phosphate uptake”. The physiological implications of this phenomenon in the phototrophic bacteria have not been elucidated, although the metabolic roles of polyPs as a high energy compound as well as a phosphate storage is proposed for some microorganisms\(^{10,16}\). Further studies on enzymes involved in polyP metabolism are being performed to find a role of polyPs in the metabolic pathways of R. sphaeroides.

References

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