Polyphosphate formation by *Acinetobacter johnsonii* 210A: effect of cellular energy status and phosphate-specific transport system

**Abstract** In acetate-limited chemostat cultures of *Acinetobacter johnsonii* 210A at a dilution rate of 0.1 h\(^{-1}\) the polyphosphate content of the cells increased from 13% to 24% of the biomass dry weight by glucose (100 mM), which was only oxidized to gluconic acid. At this dilution rate, only about 17% of the energy from glucose oxidation was calculated to be used for polyphosphate synthesis, the remaining 83% being used for biomass formation. Suspensions of non-growing, phosphate-deficient cells had a six- to tenfold increased uptake rate of phosphate and accumulated polyphosphate aerobically up to 53% of the biomass dry weight when supplied with only orthophosphate and Mg\(^{2+}\). The initial polyphosphate synthesis rate was 98 ± 17 nmol phosphate min\(^{-1}\) mg protein\(^{-1}\). Intracellular poly-β-hydroxybutyrate and lipids served as energy sources for the active uptake of phosphate and its subsequent sequestration to polyphosphate. The H\(^+\)-ATPase inhibitor N,N\(_2\) -dicyclohexylcarbodiimide caused low ATP levels and a severe inhibition of polyphosphate formation, suggesting the involvement of polyphosphate kinase in polyphosphate synthesis. It is concluded that, in *A. johnsonii* 210A, (i) polyphosphate is accumulated as the energy supply is in excess of that required for biosynthesis, (ii) not only intracellular poly-β-hydroxybutyrate but also neutral lipids can serve as an energy source for polyphosphate-kinase-mediated polyphosphate formation, (iii) phosphate-deficient cells may accumulate as much polyphosphate as activated sludges and recombinants of *Escherichia coli* designed for polyphosphate accumulation.

**Introduction**

Three enzymes have been suggested to be involved in the biosynthesis of polyphosphate: polyphosphate kinase (EC 2.4.7.1; PPK), 1,3-diphosphoglycerate: polyphosphate phosphotransferase (Kulaev 1979) and dolychylpyrophosphate:polyphosphate phosphotransferase (Kulaev 1990). Only the pathway that involves PPK has been unambiguously demonstrated in bacteria (Wood and Clark 1988; Kornberg 1995). PPK catalyses the formation of long-chain polyphosphate, up to 1000 P\(_i\) residues, in a reversible reaction: polyP\(_n\) + ATP ⇔ polyP\(_{n+1}\) + ADP. The enzyme is activated by Mg\(^{2+}\) and acts in a processive way (Ahn and Kornberg 1990; Akiyama et al. 1992).

The gene encoding PPK has been cloned, sequenced, knocked out and overproduced in *E. coli* (Akiyama et al. 1992) and *Klebsiella aerogenes* (Kato et al. 1993b). Although the gene *ppk* seems also to be present in *A. johnsonii* 210A (H.Y. Kim, personal communication), crude cell-free extracts of a wide variety of *Acinetobacter* spp. showed only low PPK activities, ranging from 0.7 to 4.7 nmol min\(^{-1}\) mg protein\(^{-1}\) (T'Seyen et al. 1985). Similar values were reported for *Acinetobacter* strains B8 and P, determined by a spectrophotometric assay method, but in extracts from other strains, including *A. johnsonii* 210A, the enzyme did not show any activity at all (Van Groenestijn et al. 1989a). Moreover, the incorporation of the terminal phosphoryl group of ATP into polyphosphate has not been demonstrated in these bacteria. This also holds for bacteria responsible for enhanced biological phosphorus removal in activated sludge (Toerien et al. 1990).
The first aim of this work was to determine the effect of the energy status of phosphate-sufficient, wild-type *A. johnsonii* 210A on its accumulation of polyphosphate during its growth in the chemostat with acetate as carbon source and glucose as auxiliary energy source. Glucose was only oxidized to gluconate by the organism when exogenous pyroloquinoline quinone (PQQ) was provided (Duine 1991; Van Veen et al. 1993a). *A. johnsonii* 210A contains two phosphate-uptake systems with $K_m$ values of 9 ± 1 μM and 0.7 ± 0.2 μM (Van Veen et al. 1993a). The constitutive, proton-motive-force-driven, low-affinity system mediated the bidirectional transport of phosphate complexed with divalent cations such as MgHPO$_4$ and CaHPO$_4$ (referred to as MeHPO$_4$) (Van Veen et al. 1993b). The binding protein and ATP-dependent, high-affinity transport system mediated the uptake of H$_2$PO$_4^-$ and HPO$_4^{2-}$ (Van Veen et al. 1994b). The synthesis of this transport system, referred to as the Pst system, was six- to tenfold stimulated by phosphate deficiency (Bonting et al. 1992a; Van Veen et al. 1993a). Therefore the potential of phosphate-deficient cells to accumulate polyphosphate was also studied, using *in vivo* $^{31}$P-NMR spectroscopy and non-growing cell suspensions. Phosphate-deficient cells were also used to produce evidence for a PPK-mediated polyphosphate formation.

**Materials and methods**

**Microorganism and cultivation**

*Acinetobacter* strain 210A was isolated from activated sludge showing enhanced biological phosphorus removal by Deinema et al. (1985), later identified as *Acinetobacter johnsonii* 210A and deposited in the Netherlands Culture Collection of Microorganisms under the access number LMAU A 130 (Bonting et al. 1992b). It was aerobically grown at 25 °C in 250-ml shaking flasks containing 100 ml medium. A culture pre-grown under similar conditions served as inoculum. This batch medium contained (g l$^{-1}$) sodium butyrate 1.14, NH$_4$Cl 0.5, MgSO$_4$ · 7H$_2$O 0.25, KH$_2$PO$_4$ 1.5, K$_2$HPO$_4$ 0.5 and 2 ml trace element solution as described by Van Groenestijn et al. (1987) (initial pH 7.0). This medium is referred to as the high-P$_i$ medium. Growth was measured by estimating the absorbance at 660 nm and the uptake of phosphate was measured by determining the phosphate concentration in the culture fluid at different times. To obtain phosphate-deficient cells, the organism was grown in the above butyrate medium with only 0.011 g KH$_2$PO$_4$ as P-source, 0.215 g KCl as K-source and 6.0 g TRIS/HCl, pH 7.2, as buffer per liter. This modified medium is referred to as the low-P$_i$ medium. The organism was unable to use TRIS as carbon and energy source was sodium acetate (2.5 g l$^{-1}$), KH$_2$PO$_4$ 1.5, K$_2$HPO$_4$ 0.5 and 2 ml trace element solution. The polyphosphate formation by non-growing suspensions of phosphate-deficient *A. johnsonii* 210A cells of 1 l culture grown in the low-P$_i$ medium were harvested by centrifugation (19 000 g, 10 min, 5 °C), washed twice with 50 mM TRIS/HCl buffer, pH 7.0, and resuspended in 100 mM TRIS/HCl buffer (containing 100 mM KCl), pH 7.0 (or 100 mM K-PIPES, pH 6.7). After addition of glucose, PQQ, KH$_2$PO$_4$ and MgSO$_4$ to final concentrations of 20 mM, 0.01 mM, 10 mM and 5 mM respectively, and 1 ml D$_2$O/10 ml suspension, polyphosphate synthesis was started by gassing the suspension with 100% O$_2$, using an air-lift system as described by Van Veen et al. (1994a). $^{31}$P-NMR spectroscopy was used to follow the formation of polyphosphate and the consumption of orthophosphate. At intervals, samples were taken directly out of the NMR tube to measure intracellular ATP (Otto et al. 1984). The H$^+$/ATPase inhibitor N,N'-dicyclohexylcarbodiimide (DCCD) was applied at a concentration of 7.5 μg mg protein$^{-1}$.

Glucose dehydrogenase activity of intact cells

The activity of glucose dehydrogenase of freshly harvested cells, grown either as batch cultures or in the chemostat, was measured with a biological oxygen monitor (Yellow Springs Instruments, Ohio, USA) at 20 °C. The cells were washed twice with 50 mM TRIS/HCl buffer (pH 7.0). A 2-ml sample of the suspension was added to 2 ml buffer with or without 4 mM CaCl$_2$. The reaction was initiated by injection of glucose to a final concentration of 25 mM. Oxygen consumption was measured polarographically with a Clark-type oxygen electrode. Oxygen uptake rates with glucose were corrected for endogenous respiration. When added to the assay mixture for glucose dehydrogenase, PQQ had a final concentration of 2.5 mM.

Total lipid extraction

Freeze-dried cells were extracted with chloroform/methanol/water (2:1:1.8, v/v/v) by the method of Bligh and Dyer (1959). The extracted lipids were then washed according to the procedure of Folch et al. (1957). The total lipid content was measured after drying under nitrogen. Fatty acid methyl esters were prepared by a 15-min incubation at 95 °C in boron trifluoride/methanol as described by Morrison and Smith (1964). The fatty acid methyl esters were extracted with hexane. PHB was extracted and quantified according to the method described by Brauneegg et al. (1978).

Fatty acid analysis

Fatty acid composition was determined by gas chromatography: with a 50-m-long capillary column model CP-Sil 88, a temperature programme of 160–220 °C, and a flame ionization detector. The instrument used was a CP-9000 gas chromatograph (Chrompack-Packard). The fatty acids were identified with the aid of standards and the relative amounts were determined from the peak areas of the methyl esters with a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan). Replicate determinations showed that the standard deviation of the values was 2%–5%. The values given represent the sum of the cis and trans isomers.

Analytical methods

Dry weight was measured after centrifugation of a 200-ml sample (19 000 g, 10 min, 5 °C), washing the pellet once with demineralized water and drying at 100 °C overnight. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Orthophosphate and the total phosphorus content of the cells were determined by persulfate digestion according to Standard Methods (American Public Health Association 1976). The polyphosphate content of the cells was calculated from the