

# Polyphosphate Accumulation by *Pseudomonas putida* CA-3 and Other Medium-Chain-Length Polyhydroxyalkanoate-Accumulating Bacteria under Aerobic Growth Conditions<sup>∇</sup>

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***Pseudomonas putida* CA-3 accumulates polyphosphate (polyP) and medium-chain-length polyhydroxyalkanoate (mclPHA) concurrently under nitrogen limitation. Five other mclPHA-accumulating *Pseudomonas* strains are capable of simultaneous polyP and mclPHA biosynthesis. It appears that polyP is not the rate-limiting step for mclPHA accumulation in these *Pseudomonas* strains.**

Certain microorganisms can accumulate energy reserve compounds, including glycogen (8, 23), lipids (1, 8), polyphosphate (polyP) (8, 11), and polyhydroxyalkanoate (PHA) (8, 13). Previous studies have reported the dual accumulation of such materials by various microorganisms (8, 28). However, the dual accumulation of medium-chain-length PHA (mclPHA) and polyP has not been reported. PolyP is a linear polymer ranging in length from 3 to more than 1,000 phosphate residues linked by high-energy phosphoanhydride bonds (11, 14, 16). Multiple roles have been suggested for polyP in the physiological adaptation of microorganisms during growth and development and in response to nutritional and environmental stress (3, 14, 16, 21). PHAs are a group of biodegradable polymers accumulated by bacteria as an intracellular carbon storage material generally in response to inorganic nutrient limitation in the presence of excess carbon (13, 25, 27). Polyhydroxybutyrate (PHB), the best-known PHA, contains four carbon monomers and is referred to as short-chain-length PHA. PHA containing monomers with six or more carbons is termed medium-chain-length PHA. A link between polyP and PHB accumulation has been widely reported in studies of phosphate removal from wastewater by enhanced biological phosphorus removal, where microbial sludges are exposed to alternating anaerobic/aerobic cycles (6, 28, 15). PHB accumulated in the anaerobic cycle is subsequently degraded in the aerobic cycle to provide energy for phosphate uptake and polyP biosynthesis. The proceeding anaerobic cycle results in the breakdown of the intracellular polyP, which provides energy for the uptake of organic substrates and results in the accumulation of PHB (12).

A search of genomic databases shows that *Pseudomonas putida* KT2440 (18) and *Pseudomonas fluorescens* PfO-1 (GenBank accession no. NC\_007492 [http://www.ncbi.nih.gov/]),

whose entire genomes have been sequenced, harbor the genes required for polyP synthesis (*ppk*) and degradation (*ppx*). However, the accumulation of polyP by these organisms has not been demonstrated. Furthermore, the simultaneous accumulation of polyP and mclPHA in any microorganism has not been reported. The successful industrial application of PHA as a biodegradable plastic is dependent on high PHA yields from bacterial fermentations. The previously reported link between PHB and polyP suggests the potential for polyP accumulation in pseudomonads to be a rate-limiting step for mclPHA accumulation by these bacteria. The present study investigates the accumulation of polyP and mclPHA by the bacterium *P. putida* CA-3 (NCIMB 41162) (19) under aerobic growth conditions to determine whether increased polyP biosynthesis could lead to increased PHA production. A range of other *Pseudomonas* strains known to accumulate mclPHA, *P. putida* S12 (9, 27), *P. putida* CA-1 (27), *P. jessenii* C8 (27), *P. fluorescens* B2 (27), and *P. putida* KT2440 (GenBank accession no. AE015451) (18), were also tested for the ability to accumulate polyP under the same growth conditions.

**Monitoring polyP and PHA accumulation over time.** *Pseudomonas* strains were grown as 400-ml batch cultures in 1-liter conical flasks containing nitrogen (N)-limited E2 medium (29) (i.e., 1.0 g NaNH<sub>4</sub>HPO<sub>4</sub> · 4H<sub>2</sub>O/liter = 67 mg N/liter) at 30°C with shaking at 200 rpm for 48 h with phenylacetic acid (2 g of carbon/liter) provided as the growth substrate. Cells were harvested by centrifugation (12,000 × *g* for 15 min at 4°C) and washed as previously described (16). The cells were passed twice through a French pressure cell (SIM Amico Spectronic Instruments) at a pressure of 1,000 lb/in<sup>2</sup> applied by a hydraulic press. Cell debris was removed by centrifugation at 25,000 × *g* for 10 min. PolyP was precipitated from the cell extract, and total intracellular polyP was determined by acid hydrolysis as previously described (16).

*P. putida* CA-3 accumulated polyP under growth conditions that also favor mclPHA accumulation. PolyP accumulated to low levels in *P. putida* CA-3 after 5 h of growth (Fig. 1 and 2). The level of polyP continued to increase over the course of the growth period until it reached a maximum level at 24 h (Fig. 2).

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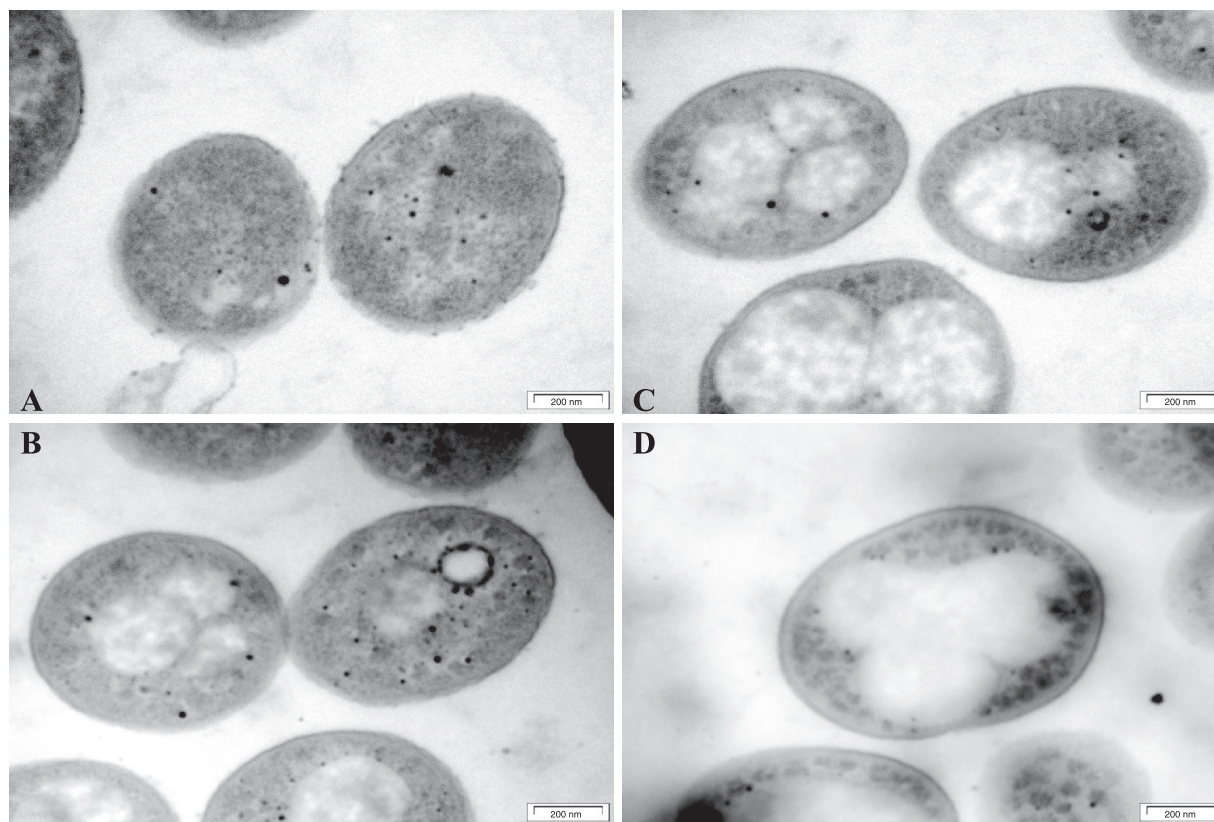


FIG. 1. Transmission electron micrograph of *P. putida* CA-3 cells containing intracellular polyP and PHA granules accumulated from phenylacetic acid harvested at various time points during growth. (A) 5 h. (B) 15 h. (C) 30 h. (D) 48 h. PolyP is visible as small black dots (inclusions). PHA is visible as large clear/white inclusions.

The concentration of N decreased rapidly over the first 5 h of growth, and a decrease in the concentration of N below 17.5 mg/liter resulted in the appearance of polyP. Interestingly, cells grown on full-strength-nitrogen E2 medium (335 mg N/liter) did not accumulate polyP after 5 h. These cells accumulated polyP to low levels after 15 h of growth, when the concentration of N in the growth medium decreased below 95.5 mg/liter. The onset of polyP accumulation by cells grown on full-strength-N E2 medium begins in the presence of much higher concentrations of N (95.5 mg/liter) than the concentration initially provided in the N-limited E2 medium (67 mg/liter). A higher cell density (1.1 g cell dry weight [CDW]) was achieved in cells grown on full-strength-N E2 medium than in cells grown on N-limited E2 medium (0.13 g CDW), suggesting a higher demand for N. Based on the CDW-to-N ratio observed at the onset of polyP accumulation in N-limited cells (0.13 g CDW:17.5 mg N), it appears that the onset of polyP accumulation should have occurred at a higher concentration of N (149 mg N/liter rather than 93.5 mg N/liter). Further studies are required to resolve this anomaly. However, it is clear that once the concentration of N becomes limiting, polyP accumulation occurs. Previous studies have reported the accumulation of polyP by *Escherichia coli* in response to nitrogen limitation (3, 11). As previously reported, negligible levels (0.3% CDW) of mclPHA were observed in *P. putida* CA-3 cells 5 h after inoculation (31) (Fig. 2). The increase in polyP and PHA levels occurred concurrently until 24 h, when the intracellular con-

centration of polyP reached a maximum level. While the level of mclPHA continued to increase after 24 h, albeit at a lower rate, the degradation of polyP was observed after this time point (Fig. 2). The decreased rate of mclPHA accumulation could be due to a limitation in reducing power, i.e., from NADH, which is required to fuel the anabolic reactions (de novo fatty acid synthesis). An initial rise in NADH levels in the cytoplasm of cells is known to stimulate PHA accumulation, and a depletion of these reducing agents over time may be responsible for the biphasic nature of PHA accumulation (2). Indeed, a threefold decrease in the concentration of polyP occurred over a 6-h period (from 24 to 30 h). However, the rate of degradation decreased after 30 h, and low levels of polyP remained in the cells after 48 h of growth (Fig. 2). While the accumulation of PHB and polyP has been reported to be cyclical, i.e., with PHB synthesis occurring in the anaerobic phase of growth and polyP synthesis occurring in an aerobic phase (6, 15, 28), it appears that the accumulation of polyP and mclPHA by *P. putida* CA-3 occurs concurrently for 19 h (Fig. 1 and 2). Thus, the simultaneous accumulation of the two biopolymers suggests that polyP is not driving the biosynthesis of PHA in *P. putida* CA-3.

**PolyP metabolic enzyme activities.** Polyphosphate kinase (PPK) and exopolyphosphatase (PPX) activities were determined according to the protocol of Mullan et al. (16, 17), using cell extracts obtained from strain CA-3 grown in E2 mineral medium with phenylacetic acid as the carbon and energy

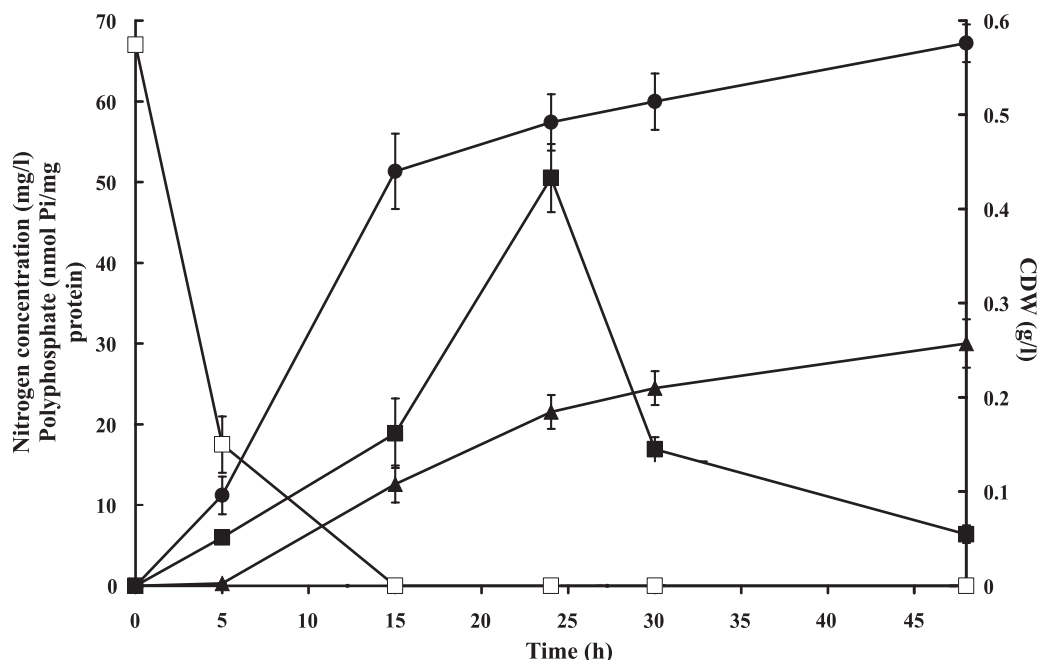


FIG. 2. PolyP biosynthesis and PHA accumulation by *P. putida* CA-3 when 2.0 g/liter of phenylacetic acid is supplied to 400 ml growth medium containing 67 mg nitrogen/liter. PolyP biosynthesis (nmol  $P_i$ /mg protein) (■), PHA accumulation (% CDW) (▲), and cell dry weight (mg/ml) (○) were monitored over a 48-h period. Nitrogen (□) depletion is expressed in mg/liter. All data are averages of at least three independent determinations.

source. Protein levels were determined using the bicinchoninic acid method (24) (Table 1). PPK catalyzes the reversible transfer of the terminal phosphate from ATP to polyP (32). Interestingly, despite the detection of polyP biosynthesis by *P. putida* CA-3, PPK levels were undetectable using the meta-chromatic assay method of Mullan et al. (15), which has a lower detection limit for PPK of 0.01  $\mu\text{mol } P_i/\text{min}/\text{mg}$  protein. Previous studies with *P. aeruginosa* (32), *E. coli* (7), and *Neisseria meningitidis* (26) have also reported that PPK activity was undetectable in cells accumulating polyP. Interestingly, PPK activity was undetectable in *Burkholderia cepacia* cells accumulating low levels of polyP at pH 7.5, but the enzyme activity was detectable at pH 5.5, when much higher levels of polyP accumulation were also observed (16). Furthermore, it has been reported that no significant amino acid homologies to PPK have been detected in a number of microorganisms capable of polyP accumulation (e.g., *Enterococcus faecalis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Haemophilus influenzae*, and *Saccha-*

*romyces cerevisiae*) (11). Indeed, PHB-calcium-polyP membrane complexes have been reported to be present in *E. coli* mutants lacking PPK (5). Consequently, it has been suggested that, for a number of strains, the intracellular accumulation of polyP could potentially involve alternative, unspecified pathways (11, 16). PPX activity, responsible for the degradation of polyP, was detected at low levels in extracts of *P. putida* CA-3 harvested at 5 h (Table 1). Enzyme activity increased in extracts of cells harvested at later time points until 30 h, when maximum activity was observed (Table 1). A 2.85-fold-lower level of PPX activity was observed in extracts of cells harvested at 48 h (Table 1). Previous reports have shown the activity of polyP-degrading enzymes to be low in the absence of polyP in the cells (4). However, upon detection of polyP, the activity of these enzymes increases significantly (4). The PPX activity in *P. putida* CA-3 shows a similar pattern (Table 1).

**PACoA ligase activity.** Some studies have shown that entry into the stationary phase of growth coincides with a decrease in intracellular polyP levels (16, 22), while others have associated bacterial survival with the accumulation of polyP in the stationary phase of growth (10, 32). To determine when the *P. putida* CA-3 cells entered the stationary phase of growth, the activity of a catabolic enzyme in the degradation of phenylacetic acid-coenzyme A (PACoA) ligase, was quantified. This enzyme is responsible for the first step in the metabolism of phenylacetic acid in *P. putida* CA-3 (20, 30). The highest rate of PACoA ligase enzyme activity was observed in extracts of cells harvested at 5 h (Table 1). All cells harvested after this time showed approximately 10-fold-lower levels of PACoA ligase activity, indicating, as predicted, that after 5 h, the cells had entered the stationary phase of growth (Table 1). Thus, in

TABLE 1. Activities of PPK, PPX, and PACoA ligase in *P. putida* CA-3 cells at various times during growth

Enzyme	Enzyme activity at indicated time (h) during growth <sup>a</sup>				
	5	15	24	30	48
PPK <sup>b</sup>	<0.01	<0.01	<0.01	<0.01	<0.01
PPX	1.71 ± 0.51	4.59 ± 1.29	8.3 ± 0.87	10.21 ± 0.16	3.58 ± 0.79
PACoA ligase	3.24 ± 0.82	0.24 ± 0.01	0.31 ± 0.04	0.31 ± 0.05	0.29 ± 0.09

<sup>a</sup> All data are averages of at least three independent determinations. Polyphosphate degradation is expressed in nmoles  $P_i$  liberated/min/mg protein. PACoA formation is expressed in nmoles PACoA formed/min/mg protein.

<sup>b</sup> The lower limit of detection for PPK activity is 0.01  $\mu\text{mol } P_i/\text{min}/\text{mg}$  protein.

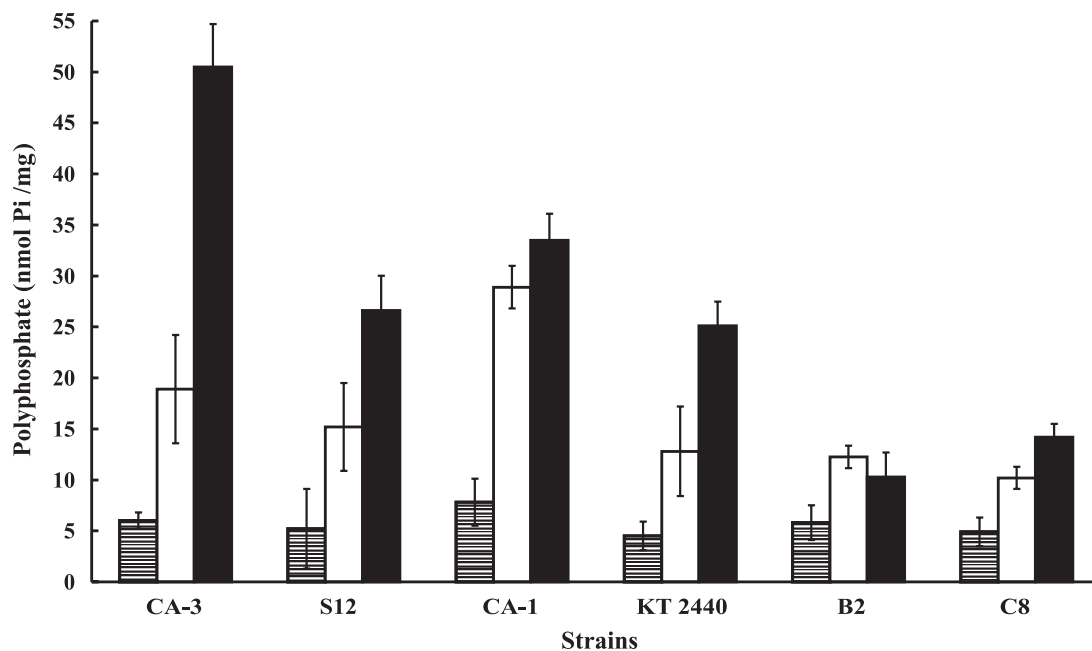


FIG. 3. PolyP accumulation by the mclPHA accumulators *Pseudomonas putida* CA-3, S12, CA-1, and KT2440; *P. jessenii* C8; and *P. fluorescens* B2. All data are averages of at least three independent determinations. Hatched bars, 5 h; open bars, 15 h; filled bars, 24 h.

contrast to previous reports on polyP accumulation by bacteria (16, 22), it would appear that both accumulation and degradation of polyP occur during the stationary phase of growth in the mclPHA accumulator *P. putida* CA-3.

**Gel electrophoresis analysis of polyP.** The chain length of polyP accumulated by *P. putida* CA-3 was monitored by gel electrophoresis as previously described (14, 16). Aliquots (30  $\mu$ l) of unhydrolyzed polyP samples were run on 15% Tris-borate-EDTA-urea gels (14, 16). Gels were stained with toluidine blue (0.05%) in 25% methanol for 15 to 20 min followed by destaining overnight in 25% methanol. The size range of accumulated polyP at selected time points was estimated by comparison to polyP standards (with chain lengths equivalent to 25, 45, and 75 residues). PolyP extracted from cells at 5 h had a low molecular weight corresponding to a chain length of 25 residues (data not shown). PolyP with the highest molecular weight, equivalent to 75 residues, was observed at 15 h. Cells harvested at 24 h contained polyP with a molecular weight equivalent to approximately 45 residues, indicating that the degradation of polyP was already occurring. This is in agreement with the high levels of PPX activity in extracts of cells harvested at 24 h. Further degradation of polyP occurred, yielding a polymer of approximately 25 residues at both 30 and 48 h.

**PolyP accumulation by other *Pseudomonas* strains.** *P. putida* KT2440 (25) and four other *Pseudomonas* strains (27) are known mclPHA accumulators. *P. putida* KT2440 harbors genes for polyP accumulation and degradation (18). Thus, the analysis of polyP accumulation by this and other known mclPHA-accumulating strains was undertaken (Fig. 3). Four of the five strains tested, strains S12, CA-1, KT2440, and C8, showed an initial trend in polyP accumulation similar to that observed in *P. putida* CA-3, where levels increased between 5 and 24 h (Fig. 3). (A decrease in polyP biosynthesis by *P. fluorescens* B2

was observed at 24 h.) The levels of polyP accumulated by all strains at 24 h are lower than those observed for strain CA-3 (Fig. 2 and 3). It is possible that the polyP levels observed in *P. putida* CA-3 and other strains could be increased by exposing the cells to other environmental signals or stresses.

While the simultaneous accumulation of mclPHA and polyP occurs in all strains tested, the exact role for polyP accumulation by *P. putida* CA-3 and other mclPHA accumulators is unclear. It is possible that the energy produced by the degradation of the high-energy phosphoanhydride bonds in the stationary phase of growth may contribute to some of the energy requirements for the continued accumulation of mclPHA where other energy resources are exhausted. Mutants devoid of the ability to accumulate and/or degrade polyP could determine the exact effect of polyP biosynthesis on mclPHA accumulation in these strains.

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