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# Genetic Improvement of *Escherichia coli* for Enhanced Biological Removal of Phosphate from Wastewater

JUNICHI KATO, KATSUFUMI YAMADA, AYAKO MURAMATSU, HARDOYO, AND HISAO OHTAKE*

*Department of Fermentation Technology, Hiroshima University, Higashi-Hiroshima, Hiroshima 724, Japan*

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**The ability of *Escherichia coli* MV1184 to accumulate inorganic phosphate ($P_i$) was enhanced by manipulating the genes involved in the transport and metabolism of $P_i$. The high-level $P_i$ accumulation was achieved by modifying the genetic regulation and increasing the dosage of the *E. coli* genes encoding polyphosphate kinase (*ppk*), acetate kinase (*ackA*), and the phosphate-inducible transport system (*pstS*, *pstC*, *pstA*, and *pstB*). Acetate kinase was employed as an ATP regeneration system for polyphosphate synthesis. Recombinant strains, which contained either pBC29 (carrying *ppk*) or pEP02.2 (*pst* operon), removed approximately two- and threefold, respectively, more $P_i$ from minimal medium than did the control strain. The highest rates of $P_i$ removal were obtained by strain MV1184 containing pEP03 (*ppk* and *ackA*). However, unlike the control strain, MV1184(pEP03) released $P_i$ to the medium after growth had stopped. Drastic changes in growth and $P_i$ uptake were observed when pBC29 (*ppk*) and pEP02.2 (*pst* operon) were introduced simultaneously into MV1184. Even though growth of this recombinant was severely limited in minimal medium, the recombinant could remove approximately threefold more $P_i$ than the control strain. Consequently, the phosphorus content of this recombinant reached a maximum of approximately 16% on a dry weight basis (49% as phosphate).**

Inorganic phosphate ($P_i$) is recognized as one of the major nutrients contributing to eutrophication of lakes, bays, and other natural waters (10). $P_i$ removal from wastewaters has been conducted by chemical precipitation with lime, alum, and ferric chloride. However, the chemical treatment methods are expensive, and this has led to the development of biological $P_i$ removal which provides an alternative to chemical treatment methods (9, 18, 19).

Many bacteria are known to be capable of accumulating excess $P_i$ in the form of polyphosphate (polyP) (16). Although the physiological functions of polyP are not fully elucidated, polyP has been implicated as (i) an energy source because of its ready conversion to ATP, (ii) a substitute for ATP in kinase reactions, (iii) a phosphate reservoir with osmotic advantages, and (iv) a component to enable the entry of DNA during the genetic transformation of several bacterial species (1).

In *Escherichia coli*, $P_i$ enters via either the high-affinity phosphate-specific transport system or the low-affinity phosphate inorganic transport system (Fig. 1) (3, 8, 20, 22). The *pst* operon consists of five genes, namely, *pstS*, *pstC*, *pstA*, *pstB*, and *phoU*, transcribed anticlockwise on the *E. coli* chromosome (3, 22). The products of the first four genes are required for the transport of $P_i$, and together with the fifth gene, they are involved in the regulation of the phosphate regulon (3, 22). An enzyme responsible for polyP synthesis is the homotetrameric polyP kinase (PPK) (1). PPK polymerizes the terminal phosphate of ATP into polyP in a freely reversible reaction ($nATP \rightleftharpoons nADP + polyP_n$). The utilization and degradation of polyP are catalyzed by polyphosphatase and by specific kinases including polyP glucokinase and polyP fructokinase (12). The *ppk* gene encoding PPK has been cloned, sequenced, and overexpressed in *E. coli* (2). The *ppx* gene, which encodes an exopolyphosphatase (PPX), also has been found downstream of the *ppk* gene, constituting a polyP operon (2a).

Under ordinary operating conditions, activated sludges are capable of removing an average of only 20 to 40% of the $P_i$ concentrations normally found in municipal wastewaters (6). The $P_i$ removal capacity of activated sludges appears to be limited by their phosphorus content, which is typically 1 to 2% on a dry weight basis (6, 9, 18). Improving the ability of bacteria to accumulate $P_i$, therefore, may contribute to excess $P_i$ removal from wastewaters. This report describes the genetic improvement of $P_i$ accumulation in *E. coli* MV1184. High levels of $P_i$ accumulation were achieved by modifying the genetic regulation and increasing the dosage of the genes involved in the key steps of $P_i$ transport and metabolism.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are shown in Table 1 and Fig. 2. *E. coli* strains were maintained on L agar supplemented with the appropriate antibiotics. The concentrations of antibiotics were 50 μg/ml for ampicillin and 25 μg/ml for chloramphenicol. When induction was required, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM.

**Genetic procedures and recombinant techniques.** Standard procedures were used for plasmid preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (21). *E. coli* MV1184 was also used as the host for plasmid construction.

**Plasmid constructions.** Plasmid pBC29, which contains the *ppk* gene of *E. coli*, was a gift from A. Kornberg (Stanford University), and the construction of this plasmid has been described elsewhere (2). The *ppk* gene was expressed from its own promoter. Plasmid pEP01 is a 5.5-kb derivative of pBluescript IIKS$^+$ and contains a 2.7-kb *Mlu*I-*Pst*I fragment

* Corresponding author.

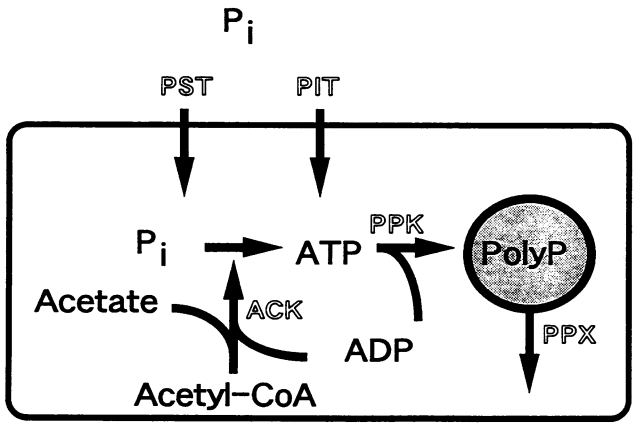


FIG. 1. Diagrammatic representation of the strategy for genetic improvement of *E. coli* for enhanced $P_i$ accumulation. Acetate kinase was employed as an ATP regeneration system for polyP synthesis. Abbreviations: ACK, acetate kinase; PIT, low-affinity system for $P_i$ transport; PST, phosphate-specific transport system; PPX, exopolyphosphatase; CoA, coenzyme A.

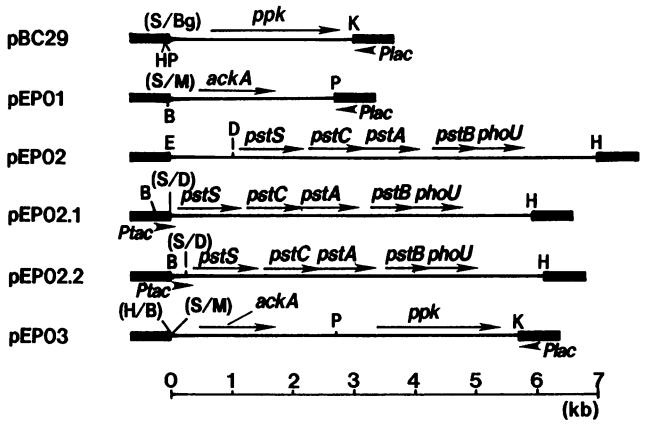


FIG. 2. Structures of recombinant plasmids used in the present study. Chromosomal inserts of plasmids are shown by thin lines, and vectors are shown as blackened lines. Arrows indicate the open reading frames and their transcriptional directions. The sites and directions of *lac* and *tac* promoters are indicated by arrowheads. Restriction site abbreviations: B, *Bam*HI; Bg, *Bgl*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sma*I. The sites in parentheses were destroyed during construction.

from pMK800 (17). This 2.7-kb fragment contains the *ackA* gene of *E. coli* and its own promoter. Although the transcriptional direction of the *ackA* gene was reverse with respect to that of the *lac* promoter in pEP01, the levels of acetate kinase activity in MV1184(pEP01) were more than 100-fold higher than the parental level.

A 7-kb *Eco*RI-*Hin*dIII fragment, which is capable of complementing the *phoS* defect in *E. coli* ANCC75, was isolated from the chromosomal DNA of *E. coli* MV1184 and cloned into pBR322 to generate pEP02. A 6-kb *Dra*I-*Hin*dIII fragment, which contains the *pst* operon, was then subcloned into the *Sma*I-*Hin*dIII site of pKK223-3 downstream from the *tac* promoter (pKP02.1). A 6.2-kb *Bam*HI-*Hin*dIII fragment was excised from this plasmid and inserted into pKTY321 to produce pEP02.2. To construct plasmid pEP03 which contains both *ppk* and *ackA*, a 2.7-kb *Bam*HI-*Pst*I fragment was excised from pEP01 and inserted at the *Hin*dIII-*Pst*I site of pBC29.

**Fractionation of cellular phosphorus.** Fractionation and estimation of cellular phosphorus compounds were carried out essentially as described by Harold (11). Cells were extracted with cold 0.5 N perchloric acid (PCA) for 20 min, with 95% ethanol (30 min at room temperature) and ethanol-ether (3:1, 1 min at 100°C), and finally with hot 0.5 N PCA (15 min at 70°C). The cold and hot PCA extracts were designated acid-soluble and -insoluble fractions, respectively. The ethanol and ethanol-ether extracts were combined and pooled as the lipid fraction. The total phosphorus of each fraction was determined by ammonium persulfate digestion; phosphate determination followed (18). Acid-sol-

TABLE 1. Bacterial strains and plasmids used

| Strain or plasmid | Relevant properties | Reference or source |
|---|---|---|
| *E. coli* strains | | |
| MV1184 | *ara*Δ(*lac-proAB*) *rpsL thi* (φ80 *lacZ* ΔM15) Δ(*srl-recA*)306::Tn*10*; Tet$^r$ F'[*traD36 proAB*$^+$ *lacI*$^q$ *lacZ* ΔM15] | 24 |
| ANCC75 | *leu purE trp his argG rpsL phoS64 met thi* | 4 |
| Plasmids | | |
| pBR322 | Cloning vector; Ap$^r$, Tc$^r$ | 5 |
| pUC18 | Cloning vector; Ap$^r$, *lacPOZ'* | 25 |
| pKK223-3 | Expression vector; Ap$^r$, p*tac* | Pharmacia |
| pBluescript IIKS$^+$ | Cloning vector; Ap$^r$, *lacPOZ'* | Stratagene |
| pKTY320 | Cloning vector; Ap$^r$, Cm$^r$, *mob*$^+$, p15A origin | 14 |
| pKTY321 | Ap$^s$ derivative of pKTY320; *Pst*I site was deleted by *Bal* 31 nuclease | This study |
| pBC29 | pUC18 harboring 3-kb *Bgl*I-*Kpn*I fragment from *E. coli*; Ap$^r$, *ppk*$^+$ | 2 |
| pMK800 | pBR322 harboring 9.5-kb *Bam*HI fragment from *E. coli*; Ap$^r$, *ackA*$^+$ | 17 |
| pEP01 | pBluescript IIKS$^+$ harboring 2.7-kb *Mlu*I-*Pst*I fragment from pMK800; Ap$^r$, *ackA*$^+$ | This study |
| pEP02 | pBR322 harboring 7-kb *Eco*RI-*Hin*dIII fragment from *E. coli* selected by complementation of *phoS* mutation in ANCC75; Ap$^r$, *pst* operon | This study |
| pEP02.1 | pKK223-3 harboring 6-kb *Dra*I-*Hin*dIII fragment of pEP02; Ap$^r$, p*tac-pst* operon | This study |
| pEP02.2 | pKTY321 harboring 6.2-kb *Bam*HI-*Hin*dIII fragment of pEP02.1; Cm$^r$, p*tac-pst* operon, p15A origin | This study |
| pEP03 | pBC29 harboring 2.7-kb *Hin*dIII-*Pst*I fragment of pEP01 | This study |

uble and -insoluble polyPs were determined by hydrolysis in 1 N HCl at 100°C for 7 min.

**Phosphate uptake experiment.** Cells were grown in 2× YT medium (21) at 37°C with shaking for 7 h, inoculated into L broth (a 1% inoculum), and incubated for 9 h under the same conditions. For cultures of recombinant strains containing pEP02.2, 0.5 mM IPTG was added to L broth before 9 h of incubation to induce the expression of the *pst* genes. Cells were transferred into T medium (13) containing 0.5 mM potassium phosphate at an optical density at 600 nm ($OD_{600}$) of about 0.2. The cell suspension was incubated at 37°C with constant stirring, and samples were taken at intervals for determination of growth and $P_i$ concentration. $P_i$ was assayed by an ascorbic acid method (18). Cell growth was determined by measuring $OD_{600}$. Cell dry weight was determined by filtering the culture sample through preweighed 0.45-μm-pore-size membrane filters and then drying them for 1 h at 103°C. A quick estimation of cell dry weight was made by using calibration curves ($OD_{600}$ as a function of dry weight).

## RESULTS

**$P_i$ removal by *E. coli* recombinants.** $P_i$ uptake experiments were performed in triplicate with strain MV1184(pUC18), a control strain, and five recombinant derivatives. Figure 3 shows a representative illustration of growth of and $P_i$ uptake by strain MV1184(pUC18) and recombinant derivatives containing pBC29 (carrying *ppk*), pEP01 (*ackA*), or pEP02.2 (*pst* operon). Growth of the three recombinant strains was almost equivalent to that of the control strain. The control strain removed about 20% of the $P_i$ from the medium during the first 3 h. However, no $P_i$ uptake occurred after growth stopped. Strains MV1184(pBC29) and MV1184 (pEP02.2) removed two- and threefold, respectively, more $P_i$ from the medium than did the control strain. Interestingly, the *E. coli* recombinant strain containing pEP02.2 (*pst* operon) alone removed more $P_i$ from the medium than did MV1184(pBC29). Strain MV1184 bearing pEP01 (*ackA*) alone took up less $P_i$, even compared with the control strain.

Strain MV1184 containing pEP03 (*ppk* and *ackA*) showed the highest rates of $P_i$ removal from the medium (Fig. 4). This recombinant strain removed approximately 90% of the $P_i$ from the medium within 4 h. Until 3 h, growth of this recombinant was almost equivalent to that of the control strain. However, unlike the control strain, the cell density of strain MV1184(pEP03) decreased after reaching a maximum at 3 h. The decrease in cell density was concomitant with an increase in $P_i$ in the medium, indicating that $P_i$ was released by cell lysis. Drastic changes in growth and $P_i$ uptake were observed when pBC29 (carrying *ppk*) and pEP02.2 (*pst* operon) were simultaneously introduced into MV1184. Growth of this recombinant was severely limited in T medium, even though this recombinant grew well in 2× YT and L media (data not shown). Nevertheless, this recombinant removed approximately threefold more $P_i$ than did the control strain. No detectable release of $P_i$ was observed with this recombinant strain. Strain MV1184 containing both pEP02.2 (*pst* operon) and pEP03 (*ppk* plus *ackA*) was also constructed. However, introduction of both of the plasmids appeared to be detrimental to MV1184. Cell viability of this recombinant decreased soon after the cells entered the stationary phase of growth in L broth. As a result, subsequent $P_i$ uptake experiments in T medium could not be performed.

**Accumulation of cellular phosphorus.** To confirm $P_i$ accumulation in bacteria, *E. coli* cells were harvested by centrifugation after 6 h of incubation, and fractionation and estimation of cellular phosphorus were carried out (Fig. 5). The results shown in Fig. 5 illustrate the amount of phosphorus of each fraction, the sum of which represents total cellular phosphorus, in MV1184(pUC18) and the five recombinant derivatives. To show the recovery of cellular phosphorus in these extracts, phosphorus content in bacteria was also estimated from the amount of $P_i$ removed and initial phosphorus content. As expected, higher levels of polyP were detected in recombinant strains, except for MV1184(pEP01), than in the control strain. Surprisingly, however, strain MV1184(pBC29 and pEP02.2) accumulated much less phosphorus than that predicted from the amount of $P_i$ removed.

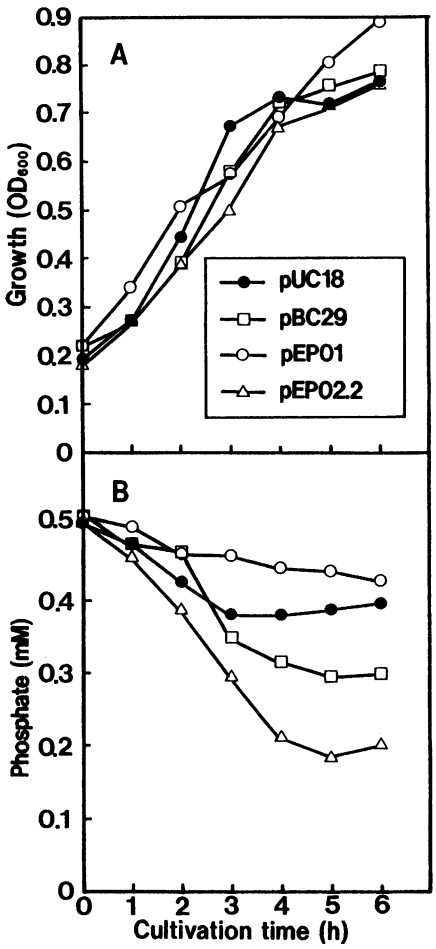


FIG. 3. Time course of cell growth (A) and $P_i$ concentration (B) during growth of *E. coli* MV1184 containing pUC18, pBC29, pEP01, or pEP02.2 in T medium containing 0.5 mM $P_i$.

To explain this discrepancy, changes in total phosphorus, as well as $P_i$, in the culture supernatants were carefully observed during the $P_i$ uptake experiment with strain MV1184(pBC29 and pEP02.2). As shown in Fig. 6, total phosphorus in the culture supernatant decreased in parallel with $P_i$ during the first 4 h. However, after 4 h, total phosphorus increased with time, even though $P_i$ still continued to decline. These results clearly indicate that phosphorus compounds, other than $P_i$, were released into the medium from bacteria. The phosphorus content in bacteria,

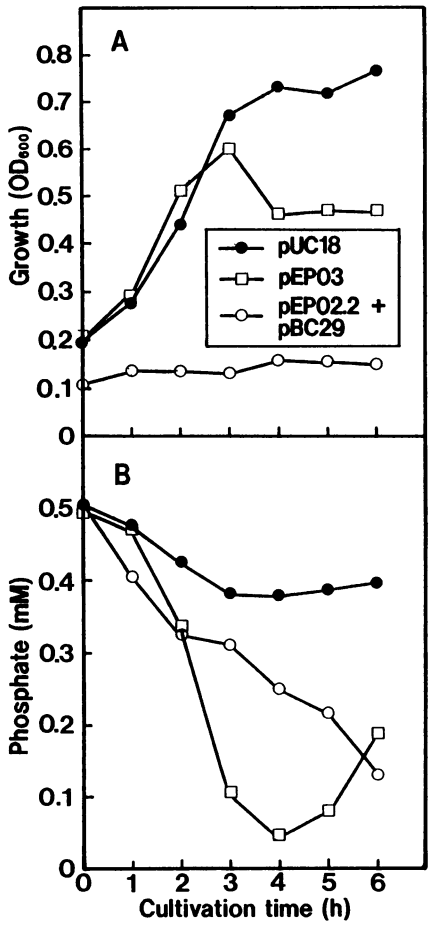


FIG. 4. Time course of cell growth (A) and $P_i$ concentration (B) during growth of *E. coli* MV1184 containing pUC18, pEP03, or pEP02.2 plus pBC29 in T medium containing 0.5 mM $P_i$.

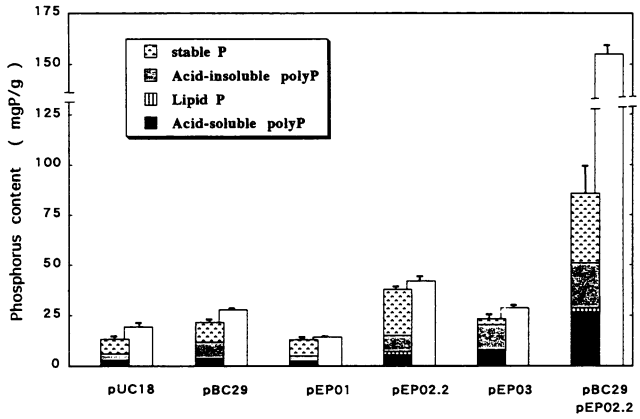


FIG. 5. Fractionation of cellular phosphorus in *E. coli* MV1184(pUC18), a control strain, and five recombinant derivatives. Cells were harvested by centrifugation after 6 h of incubation. Acid-soluble and -insoluble polyPs were determined as the acid-labile phosphorus in cold and hot PCA fractions, respectively, after hydrolysis in 1 N HCl at 100°C for 7 min. The differences between total phosphorus and acid-labile phosphorus in these fractions were defined as stable P. Lipid P was determined as the total phosphorus of lipid fraction. The open bars indicate total phosphorus which was predicted from the amount of $P_i$ removed. The results represent averages of at least three separate experiments. Error bars indicate one standard error of the mean.

calculated from the difference in total phosphorus between the cultures before and after centrifugation, reached a maximum of 16% (49% as phosphate) on a dry weight basis at 4 h but subsequently decreased to approximately 12% by 6 h. The fractionation of cellular phosphorus revealed that the content of acid-soluble plus acid-insoluble polyP decreased from 9.6% at 4 h to 8.2% at 6 h on a dry weight basis (data not shown).

## DISCUSSION

Many bacteria are capable of accumulating $P_i$ intracellularly in the form of polyP (16). Some bacterial species have also been found to take up $P_i$ far in excess of their requirements of growth after being subjected to $P_i$ starvation (12). This phenomenon, referred to as polyP overplus, is known to be caused by the elevated levels of polyP kinase (12). *E. coli* does not accumulate appreciable amounts of polyP, especially those of high-molecular-weight types, despite its possession of polyP kinase activity (15). We observed that the parental strain MV1184 did not accumulate high levels of $P_i$, even after being subjected to $P_i$ starvation (data not shown). The phosphorus content of MV1184 was approximately 1.8% on a dry weight basis, regardless of whether the cells were starved for $P_i$. This value is comparable to that of conventional activated sludge but lower than those reported in $P_i$-accumulating sludge bacteria (3 to 10% of dry weight biomass) (7, 23). Since the kinetics of bacterial $P_i$ accumulation are not fully understood, it is still difficult to specifically identify what limits $P_i$ accumulation in *E. coli*. However, in the present study, we found that the recombinant *E. coli* strain, which bears pEP02.2 (*pst* operon) alone, removed approximately threefold more $P_i$ from the medium than did the control strain (Fig. 3). The phosphorus content of this recombinant was clearly greater than that of MV1184 containing pBC29 (carrying *ppk*) alone (Fig. 5). These results suggest that $P_i$ transport may limit $P_i$ accumulation in *E. coli* MV1184. Strain MV1184(pEP02.2) appeared to accumulate more stable P than polyP (Fig. 5). However, the data are only preliminary, and further work would be needed to substantiate this point.

We attempted to improve the $P_i$ accumulation ability of *E. coli* MV1184 by modifying the genetic regulation and increasing the dosage of the genes involved in the key steps of $P_i$ transport and metabolism. As far as the phosphorus content is concerned, the best recombinant strain was MV1184(pBC29 and pEP02.2). The phosphorus content of this recombinant *E. coli* strain reached a maximum of 16% on a dry weight basis (49% as phosphate), of which approximately 60% was estimated to be stored in the form of polyP. This value was approximately 10-fold higher than that of the control strain. The bacterial ability to accumulate $P_i$ has been reported in previous papers (7, 9, 13, 18, 23). A laboratory strain of *Acinetobacter calcoaceticus* has been reported to exhibit polyP overplus, resulting in approximately 5.3% of its dry weight as phosphorus (16% as phosphate) (18). Some sludge bacteria, isolated from enhanced biological $P_i$ removal processes, were observed to accumulate as much as 10% of the biomass dry weight as phosphorus under certain conditions (23). Strain MV1184 (pBC29 and pEP02.2) surpasses these bacteria in the cellular level of phosphorus. However, it must be noted that

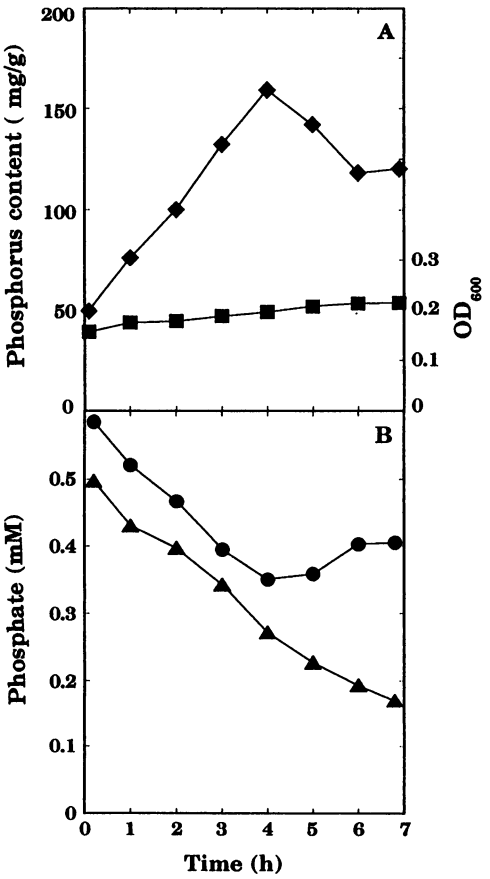


FIG. 6. Time course of total phosphorus (●) and $P_i$ (▲) concentrations in culture supernatant, cell growth (■), and total phosphorus content (◆) of MV1184(pBC29 and pEP02.2) during $P_i$ uptake experiment. Total phosphorus in bacteria was calculated from the difference in total phosphorus between the cultures before and after centrifugation. The results represent averages of experiments done in duplicate.

MV1184(pBC29 and pEP02.2) released phosphorus at a detectable amount into the medium after its phosphorus content reached a maximum. Curiously, unlike MV1184 (pEP03), this recombinant did not release $P_i$ but continued to remove $P_i$ even after phosphorus release took place. The mechanism underlying this phenomenon is unknown at the present time.

The recombinant *E. coli* strains, constructed in the present study, were capable of removing excess $P_i$ regardless of whether they were starved for $P_i$. In pBC29 and pEP03, the expression of *ppk* is still under the control of its own promoter. Pho box-like sequences have been found in the promoter (2), and the expression of *ppk* appears to be positively regulated by the products of *phoB* and *phoR* with $P_i$ starvation. Nevertheless, high levels of PPK activity were detected in MV1184(pBC29) and MV1184(pEP03), even under $P_i$-sufficient conditions (data not shown). This is attributable to the gene dosage effect because the *ppk* gene was carried on high-copy-number plasmid vectors. It would be possible to further increase the PPK enzyme activities by employing strong promoters which are not regulated by $P_i$ in the medium. Similarly, the *ackA* gene was expressed by its own promoter in pEP01 and pEP03. The exception was the *pst* operon. Increasing the gene dosage did not improve $P_i$ transport under conditions of $P_i$ excess (data not shown). In plasmid pEP02.2, the *pst* genes were inserted downstream of the *tac* promoter, and IPTG had to be added to the preculture to induce the expression of *pst* genes. We are now testing the expression of *pst* genes by various promoters which do not require IPTG.

PolyP kinase is present in numerous microorganisms (15, 16). Recently, we cloned and sequenced the PPK-encoding gene (*ppk*) from *Klebsiella aerogenes* (formerly named *Aerobacter aerogenes*). The PPK protein shares 93% identical amino acid residues with *E. coli* PPK protein. The expression of the *K. aerogenes ppk* at high levels increased polyP accumulation in this organism (unpublished data). We also detected strong hybridization signals from some sludge bacteria when the *E. coli ppk* gene was used as a DNA probe (data not shown). These results indicate the potential for genetic improvement of more useful microorganisms, including sludge bacteria, for enhanced $P_i$ removal.

In *E. coli*, further strain improvement would be possible by (i) genetic inactivation of the *ppx* gene which encodes exopolyphosphatase (2), (ii) use of more suitable promoters for the expression of *pst* genes, and (iii) optimization of the simultaneous expression of *ppk*, *ackA*, and *pst* genes. For enhanced $P_i$ removal due to engineered bacteria, the following aspects should also be investigated: (i) maintenance and stability of recombinant plasmids; (ii) effective measure for minimizing the leakage of phosphorus from cells; (iii) kinetics of $P_i$ transport and metabolism; and (iv) effects of environmental conditions such as temperature, pH, oxygen concentration, and medium composition.

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