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Expression of Polyphosphate Kinase Inhibits the Glucose Uptake in *Escherichia coli*

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ABSTRACT

This paper examines the effects of phosphate pool and expression of polyphosphate kinase on glucose uptake by expressing the polyphosphate kinase under the control of *lac* promoter. The *E. coli* transformant of pL1, containing an IPTG controllable element for polyphosphate kinase expression, showed that the total intracellular phosphate significantly increased. However, the rate of glucose uptake by the resting plasmid-bearing cells with IPTG induction significantly decreased. These findings suggest that the polyphosphate can not directly function as an energy source in *E. coli* or at least not as a good energy supplier.

Key Words: polyphosphate; glucose uptake; *E. coli.*

I. Introduction

Polyphosphate, known as volutin granules found in many bacteria, fungi, protozoas, plants, marine sponges and even mammals, is a linear polymer of orthophosphates joined by ester bonds (Booth and Gaidotti, 1995; Kumble and Kornberg, 1995; Lorenz *et al.*, 1995; Takahashi *et al.*, 1995; Yang *et al.*, 1995). Its chain can be as long as thousands of orthophosphates. However, details regarding its physiological roles in the cells remain unclear, although scientists proposed that the polyphosphate might serve as an energy source, phosphate reservoir, donor for sugar and adenylate kinase, chelator for divalent cations, buffer for alkaline stress, regulator of transcription, and/or competence component for DNA entry (Kornberg, 1995; Sharfstein and Keasling, 1994).

Previous work investigated the biochemical characteristics of polyphosphate kinase (PPK) and its genes from some bacterial strains were successfully cloned and expressed (Akiyama *et al.*, 1992; Bark *et al.*, 1993; Crooke *et al.*, 1994; Haeusler *et al.*, 1992; Hardoyo *et al.*, 1994; Kato *et al.*, 1993a, b; Tinsley and Gotschlich, 1995; Tinsley *et al.*, 1993). After comparing with their gene homology, these investigations conferred that the polyphosphate kinase genes in different groups of bacteria are evolutionarily related. The membrane-bound polyphosphate kinase from *E. coli* was a homo-tetramer and used ATP as substrate to construct the orthophosphate polymers (Akiyama *et al.*, 1992). This reaction was reversible in vitro (Hardoyo *et al.*, 1994). Nevertheless, *in vivo* reversible reaction has not yet been demonstrated.

During phosphate starvation, the intracellular polyphosphate was undetected in *E. coli* W3110. The activities of exopolyphosphatase (PPX), in which the gene was located downstream of *ppk* in *E. coli*, can hydrolyze the polyphosphate to orthophosphate; in addition, alkaline phosphatase in starvation phase was higher than those during exponential growth. During a shift from phosphate starvation to phosphate surplus condition, PPX activity decreased and PPK activity and intracellular polyphosphate content increased dramatically (Sharfstein and Keasling, 1994). However, another study reported contradictory effects of phosphate level on polyphosphate transport and *ppk* transcription expression. The expression of lacZ from the *ppk* promoter of *K. aerogenes* ATCC9621 was increased in *E.*

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coli MV1184 under conditions of phosphate limitation, but not in a *pho*B⁻ negative strain. According to their study, the *ppk* promoter is regulated by the *pho*B product (Kato *et al.*, 1993b). Such conflicting results may indicate that the accumulation of polyphosphate depends upon overall outcome from phosphate uptake, enzyme activities, expression of PPK and PPX, and availability of intracellular energy (Akiyama *et al.*, 1993; Hardoyo *et al.*, 1994; Lorenz *et al.*, 1995). The detail still needs to be solved.

In *Actinomyces naeslundii* glucose was uptaken by being phosphorylated to glucose 6-phosphate by a glucokinase that utilized polypho-sphate and GTP instead of ATP as a phosphoryl donor (Takahashi *et al.*, 1995). To our knowledge, the effects of polyphosphate on the glucose uptake in *E. coli* have never been examined. This paper demonstrates the effects of polyphosphate kinase and polyphosphate on the glucose uptake rate.

II. Materials and Methods

1. Strains and Medium

The bacterial strain *E. coli* JM109 was used as the cloning and expression host. Luria and Bertani broth (DIFCO) was used to grow the bacterium at 37 °C. In the expression phase, the bacteria were cultivated in M9-glycerol medium in which one liter of medium contained Na_2HPO_4 17.1 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, 2 ml of 1M $MgSO_4$, 0.1 ml of 1M $CaCl_2$, 5 ml of 100 g/l of proline and thiamin, and 10 g of glycerol (Sambrook *et al.*, 1989), and a final concentration of 1 mM IPTG (Sigma) was added to induce the expression of *lac*-promoter controlling genes at the cell density of 0.25-0.3 at OD600nm. After harvested by centrifuging at 3000 rpm in four-hour induction, the cells were used for total phosphorus content, SDS-PAGE, NADH oxidase activity, and glucose uptake experiments.

Plasmid, pBC29, was obtained from Drs. M. Akiyama and E. Crooke in Professor A. Kornberg's laboratory (Akiyama *et al.*, 1992, 1993). All restriction enzymes were bought from Boehringer Mannheim Co. and the reactions were carried as in the manufacturing manual. T4 DNA ligase was obtained from New England Biolab. GENECLEAN II kit (BIO 101, San Diego, USA) was used to isolate the DNA fragments from the agarose gel. NADH and glucose 510A kit were purchased from Sigma Co.

2. Cloning Strategy

A 2.9 kb DNA fragment from pBC29 digested by *Xba* I and *Kpn* I was inserted into pUC19 with the same cutting sites. After ligation, the subsequent plasmid pL1 DNA was transformed into *E. coli* JM109 strain and prepared for non-radioactive dot blotting. The restriction enzyme mapping was also performed to confirm the expected construction (Fig. 1). The detail cloning techniques and SDS-PAGE protein gel were followed as described previously (Sambrook *et al.*, 1989). A non-radioactive method for dot blotting was implemented as described in the manufacturing manual (Non-radioactive labeling and detection of nucleic acids kit Cat#1093657, Boehringer Mannheim). In brief, the isolated polyphosphate kinase gene from pBC29 was used as a template for random primed labeling with DIG-dUTP-containing mixture. Alkaline phosphatase conjugate anti-digoxigenin(DIG) antibody, X-phosphate, and NBT were used to colormetrically detect the presence of the polyphosphate kinase gene.

3. NADH Oxidase Activity

One milliliter of cell broth was washed with 0.85% NaCl and resuspended in 100 µl TE buffer (pH 8.0). The mixture was sat on ice for ten minutes after ten microliter of lysozyme was added. The cell lysate was adjusted to around 0.1 of OD600nm and scanned at optical decrease at wavelength of 340 nm after 200 µl of 1 mM NADH in Tris/HCl (pH 7.5) solution was added. The specific activity of NADH oxidase was defined as one unit of optical density decrease per milligram of protein in one minute. The relative specific activity of NADH oxidase was defined as the specific activity of induced cells divided by that of uninduced cells.

4. Phosphate Measurement

The harvested bacteria were acid digested in an autoclave at 121°C (Greenberg *et al.*, 1985). One milliliter of digested lysate after 30 min cook was thoroughly mixed with 40 µl of 2.5% ammonium molybdate dissolved in 28% sulfuric acid reagent and 10 µl of 2.5% stannous chloride reagent. The reaction was performed at room temperature for 10-12 minutes. The developed color was measured immediately by a spectrophotometer at wavelength of 690 nm. The phosphorus content was defined as the total amount of phosphate in the IPTG-induced cells divided by that of uninduced cells at each sampling time.

5. Glucose Uptake Rate

The bacteria were cultivated with or without IPTG induction as indicated in the text. The cells were harvested and washed twice with 0.85% NaCl. The

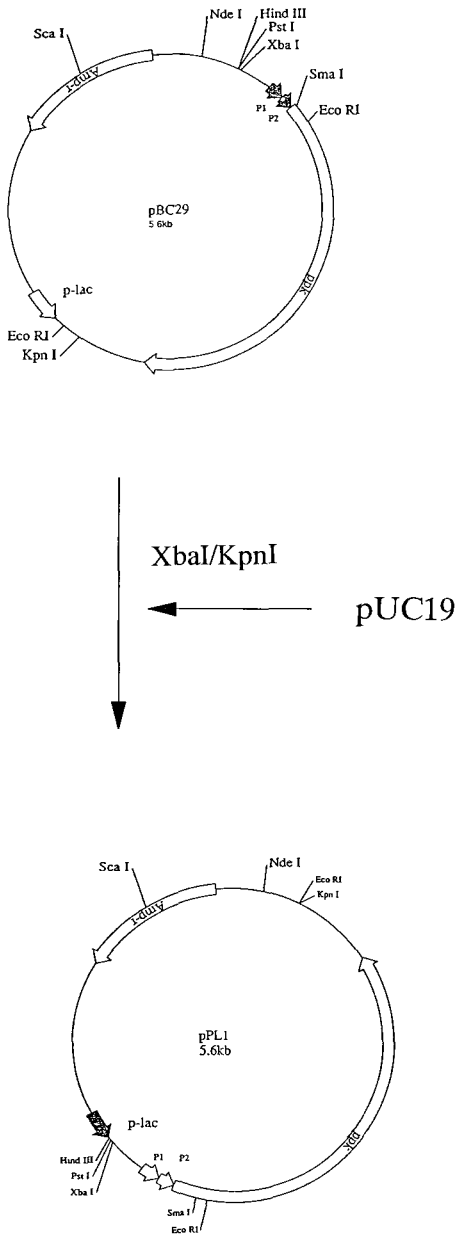

Fig. 1. Strategy of cloning for plasmid containing *lac*-controlling polyphosphate kinase. Around 3.0 kb fragment isolated from *Xba* I-*Kpn* I digested pBC29 was ligated into restricted pUC19, resulting in pL1 contained *ppk* gene under the control of *lac* promoter.

phosphate buffer (pH 7.0) was used for the glucose uptake experiments. The glucose concentration of 0.1 g/l was initially added. The reactions were performed at 37°C and the samples were taken to measure the glucose concentrations by using a Sigma glucose kit (510A). The glucose disappearance rates were used to represent the uptake rates.

III. Results

1. Clone of pL1

As Fig. 2 reveals, the subsequent plasmid, pL1, had different maps of restriction enzymes from the original pBC29. The pL1 digested with *Pst* I and *Nde* I resulted in 2.3 and 3.2 kb fragments while the expectedly digested fragments of pBC29 were 0.3 and 5.3 kb. The dot blotting revealed that the polyphosphate kinase genes were present in both pL1 and pBC29 plasmids (Fig. 3). In SDS-PAGE gel analysis, the band intensity of a 69 KD protein was more prominent in strains containing pL1 with IPTG induction than those without induction and the original clone, pBC29 (Fig. 4). There was some basal level of expression of polyphosphate kinase in the pL1 clone due to that the original promoter of polyphosphate kinase was not digested out of the plasmid. An additional band of 24 KD appeared because the translation from initial threonine of the original *lacZ* gene included parts of polyphosphate promoter region.

2. Phosphate Accumulation

As Fig. 6 reveals, the intracellular phosphate concentration per unit of total protein increased 2.2 times in the pL1-containing bacteria after IPTG induction while no significant difference arose between pUC19

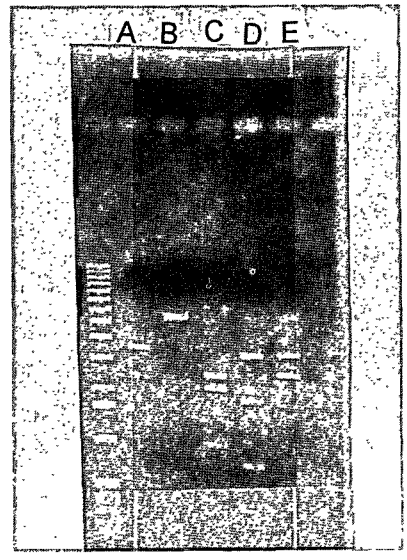

Fig. 2. Restriction enzyme mapping of pL1. Lane A: Uncut DNA; lane B: Sma I digested fragment; lane C: *Pst* I-*Nde* I-*EcoR* I; lane D: *Pst* I-*Nde* I-Sca I; lane E: Pst I-Nde I digested fragments. The molecular marker of DNA on the left-side of lane A was GIBCO BRL KB ladder marker.

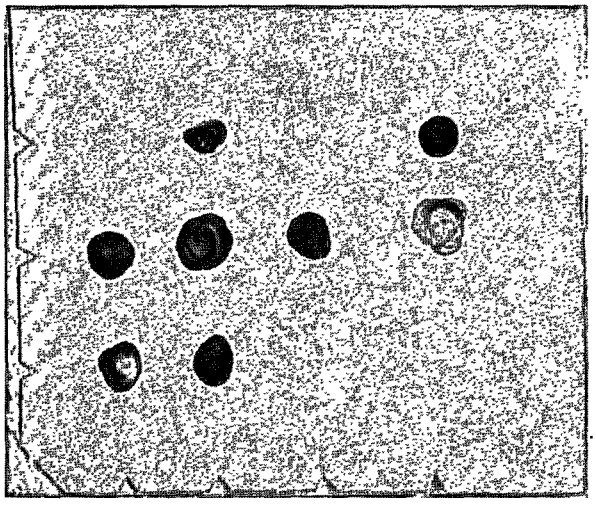

Fig. 3. Dot blot analysis for pUC19, pBC29, and the new clones, pL1 and pL2. Figure 1 depicts the map of pUC19, pBC29 and pL1. The control plasmid pL2 was same as pL1 but contained the *ppk* gene in a reversed direction which was different from promoter *lac*.

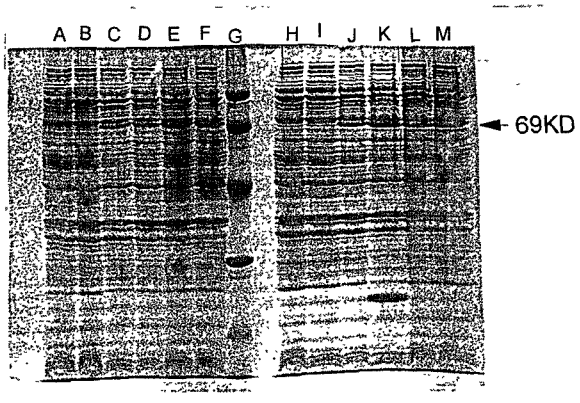

Fig. 4. Polyphosphate kinase expression of the various clones with IPTG induction. Lanes A through F were the samples taken at the time when the IPTG was added while lanes H through M were taken in four hours after IPTG addition. Lanes A and H: pUC19-containing cells without adding IPTG; lanes B and I: pUC19-containing cells with IPTG addition; lanes C and J: pL1-containing cells without IPTG addition; lanes D and K: pL1-containing cells with IPTG addition; lanes E and L: pBC29-containing cells without IPTG addition; lanes F and M: pBC29-containing cells with adding IPTG; lane G: KB ladder molecular marker.

and pBC29-containing strains. In the time courses of cell growth, the growth rates of pUC19- and pL1-containing strains were inhibited after IPTG addition while the cell growth of pBC29-containing strain was unaffected (Fig. 5). The reason for no effect is because that the pUC19- and pL1-containing strains produced the

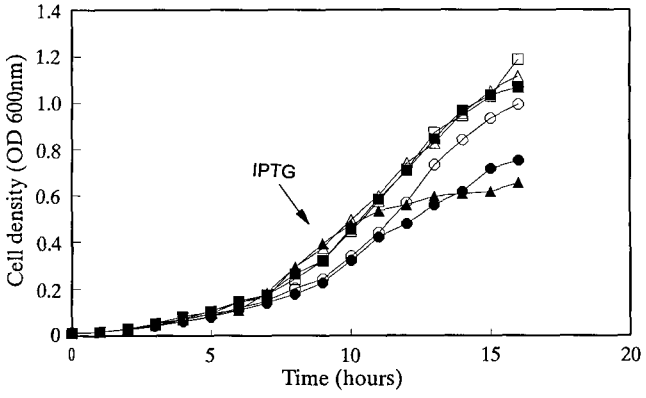

Fig. 5. Time courses of cell growth of various clones. The cells were cultivated in M9-glycerol medium. Triangles denote pUC19-containing cells; circles for pL1-containing cells; squares for pBC29-containing cells. The open symbols represent for not adding IPTG; closed symbols for IPTG addition. The IPTG was added into medium as indicated by the arrow.

recombinant proteins under the control of lac promoter while the direction of transcription of polyphosphate kinase was opposite to lac promoter in pBC29. Under IPTG induction, the pUC19-containing cells produced w subunit of *lacZ* and pL1 produced the polyphosphate kinase

3. NADH Oxidase Activity

As indicated in Fig. 7, the NADH oxidase activity was not significantly different between the pL1- and pBC29-containing strains although the specific NADH oxidase activities varied between 0.5-1.5. Because the NADH oxidase activity represents the energy flow in the electron transport system, these results indicated that the expression of polyphosphate kinase in pL1-containing strain did not affect the energy generation via electron transport chain. Nevertheless, the ATP was expected to be consumed by the polyphosphate kinase to polymerize the orthophosphate.

4. Glucose Uptake

As Fig. 8 indicates, glucose concentration decreased very rapidly to zero in 30 minutes for the backbone-plasmid pUC19-bearing *E. coli* strain. Glucose concentrations decreased to zero in one hour for pBC29-containing cells while those decreased to zero in two and four hours for uninduced and induced pL1-containing cells, respectively. The disappearance rate of glucose for pL1-containing bacteria was much slower than that for the pBC29-containing bacteria. The glucose uptake rate for IPTG-induced pL1-containing

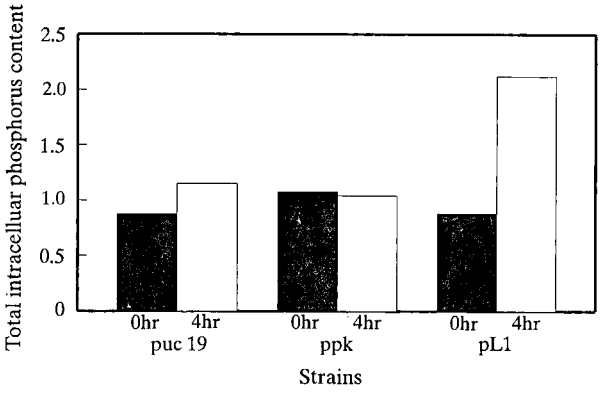

Fig. 6. Phosphorus content in various clones. The closed bars represent the phosphorus content ratio at the time when the IPTG was added while the open bars are for phosphorus content ratio in four-hour IPTG induction.

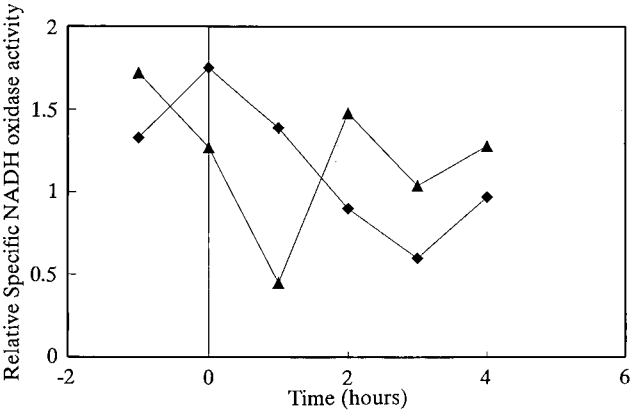

Fig. 7. Specific NADH oxidase activity in various clones. Diamonds represent the pBC29-containing cells; triangles for pL1-containing cells.

bacteria was lower than that without IPTG induction. Notably, glucose uptake rate for pL1-containing bacteria under no induction condition was only slightly higher than the rates for pBC29-containing strains under either IPTG induction or no induction.

IV. Discussion

This study has demonstrated that the glucose uptake rate significantly decreased once the polyphosphate kinase was expressed (Fig. 6). Meanwhile the energy-generating system, NADH oxidase was maintained constant working regardless of what the gene expression was (Fig. 7). We can conclude that ATP depleted by polyphosphate kinase limited the energy for the glucose uptake. Although the role of polyphosphate on the polyphosphate glucokinase and fructokinase has been suggested, the glucose uptake results indicate that no such activity in *E. coli* exists.

For the operation engineers of waste water treatment plants, several critical factors should be considered for efficient phosphate removal using wild type strains (Ahring *et al.*, 1993; Jogensen and Pauli, 1995). The phosphate removal rate depends proportionally on both the high concentrations of phosphate and enzyme; meanwhile, the enzyme expression was repressed in high phosphate concentration. Therefore, phosphate removal efficiency by microorganisms with negative regulation, in which polyphosphate kinase production was favored at the low concentration of phosphate (Bond *et al.*, 1995; Crooke *et al.*, 1994), was a trade-off between phosphate concentration and amount of expressed enzyme. Based upon our recommendation, environmental engineers were requested to additionally consider ATP energy balancing in employed bacteria for the phosphate removal process operation.

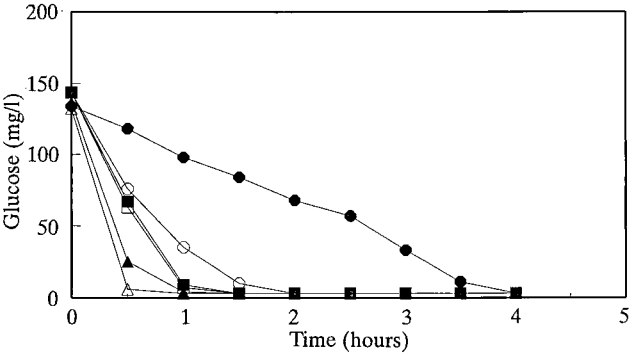

Fig. 8. The effects of polyphosphate kinase expression on the glucose uptake rates in various clones. The closed symbols represent the cells with IPTG induction; open symbols for no IPTG induction. The circles denote pL1-containing cells; squares for pBC29-containing cells; triangles for pUC19-containing cells.

Concentration of carbon source should be adequately controlled at a sufficient level. Otherwise, the cells would not uptake the sugar to generate ATP for polyphosphate formation once the ATP was depleted by polyphosphate kinase. This phenomenon caused an unstable situation in the waste treatment process, usually occurring in the municipal waste plants.

In summary of the results for the polyphosphate roles in *E. coli* physiology, we propose a mechanism in which the polyphosphate kinase consumed ATP and competed with the glucose uptake system, EIIIGlc. Therefore, the energy become less inside the cells containing a large amount of polyphosphate material and polyphosphate kinase. This proposed mechanism clari-

fies why the waste-treatment using natural phosphorus-accumulating bacteria is not in efficient, suggesting that the energy-generating system should be employed to enhance the phosphate removal efficiency.

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聚磷酸酵素的表現抑制大腸桿菌攝取葡萄糖

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摘　要

本研究爲避免生物本身的磷調節作用干擾此酵素的產生量及胞內磷酸鹽的蓄積量，我們將聚磷酸酵素的操作子換成乳糖的操作子而得到pL1質體。以IPTG誘導含pL1質體的大腸桿菌，結果使得菌體內總磷酸含量大增，然而卻降低此菌的葡萄糖攝取力。由此結果可知，聚磷酸鹽並無法直接當作糖代謝酵素的能量供應者。