Korean Journal of Life Science
Vol. 10. No. 4. 397~402, 2000

Characterization of Polyphosphate Kinase Gene in *Serratia marcescens*

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Abstract

Polyphosphate kinase catalyzes the formation of polyphosphate from ATP. To understand the mechanism of phosphate accumulation, the *Serratia marcescens* gene encoding *ppk* was cloned from the genomic library by the method of Southern hybridization. The hybridization positive DNA fragment region from pDH3 was subcloned into the expression vector. The *ppk* gene product, a polypeptide of 75 kDa, was confirmed by SDS-PAGE. Expression of the *Serratia marcescens ppk* gene is regulated by the catabolite repression system. The enzyme activity polyphosphate kinase was increased in the *E. coli* strain harboring plasmid pMH4 with *ppk* gene.

Key words – *Serratia marcescens, ppk* gene, Polyphosphate, Gene cloning, Gene expression

Introduction

Many organisms store energy and phosphate in polymers of 3 to more than 1000 phosphate residues called polyphosphate(polyP) [23]. It is found in a wide variety of organisms, including bacteria, fungi, protozoa, plants and mammals [15]. Inorganic phosphate (Pi) is recognized as one of the major nutrient contributing to eutrophication of lakes, bays and other natural waters [10]. Pi removal from waste waters 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 Pi removal which provides an alternative to chemical treatment methods [18].

Many bacteria are known to be capable of accumulating excess Pi in the form of polyphosphate [14]. Although the physiological function 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 reation, (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 [1,13]. PolyP is built up from ATP by polyphosphate kinase (Ppk) in a reversible and highly processive reaction from a phosphohistidyl-Ppk intermediate [16]. Recently, the *ppk* gene encoding PPK has been cloned and analyzed in some bacteria, *E. coli* [2], *Klebsiella* sp. [12], *Acinetobacter* sp. [9] and *Pseudomonas* sp. [4]. However, no investigation has been undertaken to identify and characterize the genes involved in the polyP metabolism in *Serratia* sp.

The understanding of biochemical mechanism and genetics of bacterial phosphorus transport and metabolism is essential for improving their abilities to remove phosphorus from waste waters [19]. The aim of this study was to analysis the gene responsible for polyphosphate synthesis in *Serratia marcescens*.

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Materials and Methods

Bactarial strains, plasmids and culture conditions

S. marcescens KCTC 2172 strains was purchased from Korea culture type collection [8]. The *E. coli* strains were JM109 and TP2139(M. Casadaban). pBluescript KS (+) and pKK223-4 were used for cloning vectors. Bacterial strains were routinely grown at 30 or 37℃ and maintained. The antibiotic used was: 50 ㎍/㎖ ampicillin. Cultures for enzyme assay were grown in minimal medium with 10 mM potassium acetate and 0.3% casamino acids.

DNA isolation and manipulation

Total cellular DNA from *S. marcescens* was prepared as previously described [24]. Rapid, small-scale plasmid DNA isolation was performed by the method of Birnbaum and Doly [6] and Wizard kit of Promega biotech. All restriction enzymes, T_4 DNA ligase, and polynucleotide kinase were obtained from Takara shuzo Co. and were used according to the recommendation of the suppliers. General recombinant DNA manipulation was carried out according to the protocol suggested by Sambrook *et al.* [21].

Preparation of cell extracts and enzyme assays

The cultured cells were harvested and suspended in cell suspension buffer (50 mM MOPS, pH 7.0, 0.5 M $(NH_4)_2SO_4$, 20 %[vol/vol] glycerol, 1 mM EDTA). The suspension was sonicated and cell debris was removed by centrifugation. The protein content of the cell extracts was determined by the method of Barford [5] with bovine serum albumin as the standard (Bio-Rad). The activity assays for polyphosphate kinase was carried out by the hydroxamate methods of Rose. [20] The assay mixture contained(in 1㎖) 0.78 M tripolyphosphate, 48 mM Tris-HCl, 10 mM $MgCl_2$, 0.7 M KOH, 5% (V/V) hydroxylamine hydrochloride, and 150 ㎕ of crude extract. The reaction was initiated by the addition of ATP to a final concentration of 10 mM and proceeded for 5 min at 29℃. The reaction was stopped by the addition of 1 ㎖ of 10% trichloroacetic acid. The quentity of the end product was determined by the addition of 4 ㎖ $FeCl_3$ reagent. The dbsorbance is read at 540 nM.

Southern hybridization

Total cellular DNA from *Serratia marcescens* digested with several restriction enzymes. The digested DNA was electrophoresed on a 0.7% agarose gel, transferred onto a NC filter [22], and then hybridized with *E. coli* probe [9].

The 2 kb DNA probe, which was obtained from PCR with the genomic DNA of *E. coli* as the template. The nucleotide sequence of the *E. coli ppk* specific primers for PCR were p1: 5'TGGAGAATTCATGGGTCAGG-AAAGCTTCT3' and p2: 5'ATAGAAGCTTGCCTACTGTCCTGGTTG3'. Double strand DNA probe was prepared by using the random primer DNA labeling system with [α-^{32}P]dCTP [3].

SDS-PAGE

The crude protein extracts were prepared cells grown over night in M9-medium with 0.3% casamino acids. The cells were suspended in cell suspension buffer(50 mM Tris-HCl buffer, pH 7.5). The cell suspension was sonicated, and cell debris was removed by centrifugation. The crude cell proteins were fractionated on 10% SDS- polyacrylamide gel and stained with Coomassie Brilliant Blue.

The crude cell proteins were seperated by SDS-polyacrylamaide gel electrophoresis by the method of Laemmli [17].

Results and Discussion

Cloning of the *Serratia marcescens ppk* gene

For cloning the complete *ppk* gene, genome DNA of *S. marcescens* was digested with several restriction enzymes and hybridized with a 2 kb probe DNA of *E. coli ppk* region. Hybridization experiments using an *E. coli ppk* probe [2] showed a 8.5 kb *Hin*dIII positive DNA fragment of *Serratia marcescens* genomic DNA to be the most suitable for cloning (Fig. 1). Therefore, in order to clone the positive DNA

fragment, *Hind*III-digested genomic DNA fragments with the size of 7.5~9.5 kb were fractionated from 0.7% agarose gel and ligated with the vector pBluescriptII KS (+) digested with *Hind*III. *E. coli* JM109 comepetent cells were transformed with the ligation mixture and spread onto LB plates containing 0.5 mM IPTG/50 μg per ml of X-gal/50 μg per ml of ampicillin. Plasmid DNAs, isolated from one thousand recombinant colonies of white color, were hybridized against the probe DNA, one positive plasmid was obtained. The recombinant plasmid DNA has 8.5 kb fragment and was designated as pDH3. The clone was digested with several restriction enzymes to obtain a restriction map of pDH3 (Fig. 2). Restriction map and Southern hybridization analysis revealed that the 2.0 kb *Bam*HI fragment of pDH3 contained the sequence of *ppk* gene (Fig. 3). Therefore, the 4.0 kb *Mlu*I-*Hind*III fragment of pDH3 was blunt-ended by Klenow fragments and subcloned into pBluescript (KS+) digested with *Eco*RV. This recombinant plasmid was designated pMH4. *E. coli* containing the recombinant plasmid pMH4 had increased enzyme activity of polyphosphate kinase. These results suggested that the DNA fragment of pDH3 is sufficient to encode the *ppk* gene.

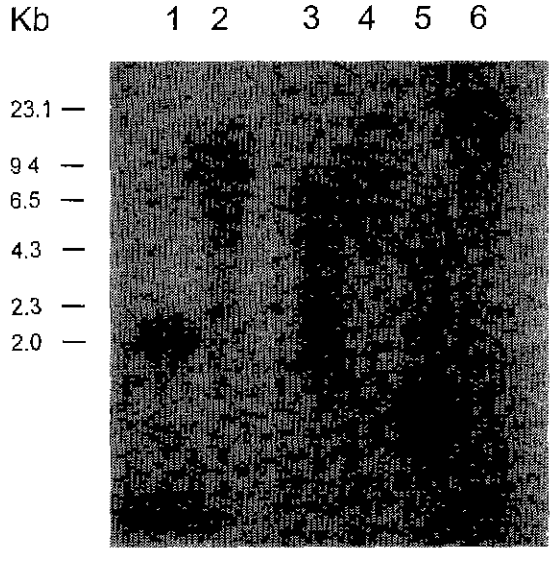

Fig. 1. Southern blot hybridization of *S. marcescens* chromosomal DNA with *ppk* probe DNA of *E. coli*
S. marcescens chromosomal DNA samples were digested with several restriction enzymes, separated on 0.7% agarose gel and transferred onto nylon membrane. The genomic DNA was hybridized with probe DNA labeled [α-^{32}P] dCTP. Lanes: 1; Digested by *Bam*HI, 2; *Eco*RI, 3; *Hind*III, 4; *Kpn*I, 5; *Pst*I, 6; *Sal*I.

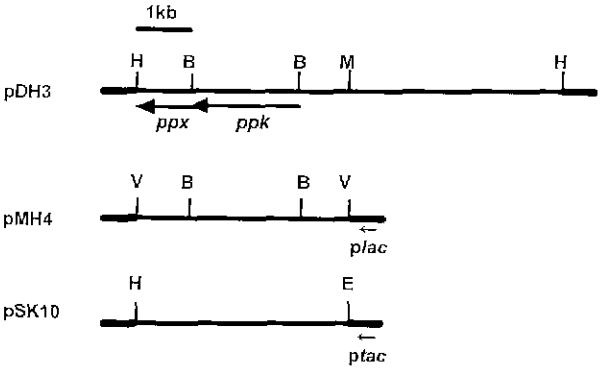

Fig. 2. Physical map of DNA fragments from *S. marcescens* cloned in plasmid.
The initiation site and direction of transcription of *ppk*, *ppx* genes, p*tac* and p*lac* are shown Abbreviations: B; *Bam*HI, E; *Eco*RI, H; *Hind*III, M; *Mlu*I, V; *Eco*RV.

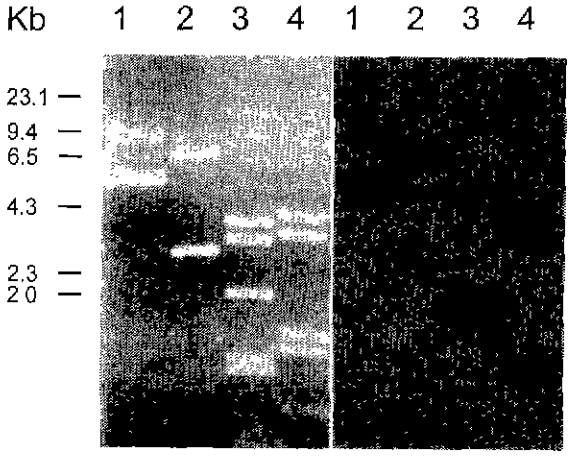

Fig. 3. Southern blot hybridization of recombinant DNA pDH3 with *ppk* probe DNA of *E. coli*
Recombinant DNA sample were digested with several restriction enzymes, separated on 0.7% agarose gel and transferred onto nylon membrane.
Lanes: 1. pDH3, 2: pDH3/*Hind*III, 3: pDH3/*Bam*H I, 4: pDH3/*Pst* I

Enzyme activity of polyphosphate kinase in *Serratia marcescens*

The enzyme activity of polyphosphate kinase was ob-

Table 1. Polyphosphate kinase activities of crude extract from *Serratia marcescens*

Sugar	Specific Ppk activity[1)]
glucose	0.06
lactose	0.14

Cell were grown on M9 media containing glucose or lactose
[1)]One unit of enzyme is defined as the amount which produced 1 μmole of hydroxamic acid per min.

served in *Serratia marcescens* cells. We have also determined the levels of polyphosphate activity in order to determine whether the expression of *ppk* gene was regulated by catabolite repression system [7]. As shown in Table 1, the activity of polyphosphate kinase is 2.3-fold higher in the presence of lactose for carbon source than that in the presence of glucose. This result indicates that cAMP-CRP complex may be required for the expression of *Serratia marcescens ppk* gene.

Expression of the *S. marcescens* ppk gene in *E. coli*

In order to find whether the increased expression of *ppk* under the direction of a strong promoter such as *tac* promoter is necessary for the induction of polyphosphate kinase. Genes for the putative of *S. marcescens* polyphosphate kinase were introduced into the bacterial expression vector pKK223-4. The putative polyphosphate kinase gene region of pMH4 was cut with *Eco*RI-*Hind*III and ligated into the same site of pKK223-4 to construct pSK10. The basal enzymatic activity was determined using cellular lysates of *E. coli* containing pKK223-4 as a control. Cells were transformed with the plasmids pMH4, pSK10, pBluescript KS(+) and pKK223-4 to test for the expression of polyphosphate kinase. In cells harboring either pMH4 or pSK10, but not the vector plasmid, the enzyme activity increased after induction. *E. coli* crude lysates expressing pSK10 showed a 16-fold increase in enzyme activity(Table 2). In plasmid pSK11, the cloned gene is in the opposite direction and therefore is transcribed in the reverse direction to pSK10, it showed low level activity as the same set of *E. coli*. Expression of polyphosphate kinase was

Table 2. polyphosphate kinase activites of *E. coli* containing the recombinant plasmides

plasmides	specific ppk activites[1)]
pBluescript KS(+)	0.042
pDH 3	0.245
pKK 223-4	0.045
pSK 10	0.690

Cells were grown on M9 media at 23℃.
[1)]One unit of enzyme is defined as the amount which produced 1 μmol of hydroxamic acid per min.

independent of the orientation of the *Mlu*I-*Hind*III fragment of pDH3 contained the sequence of *ppk* gene, which is indicated that it contains all essential promoter elements.

After induction of polyphosphate kinase with isopropyl-*thio*-β-D-galactopyronoside, crude extracts were prepared from cultured bacterial cells and were analyzed by SDS-PAGE. As shown in Fig. 4, distinct bands corresponding to proteins of approximately 75 kDa was detected by SDS-PAGE. In *E. coli* and *Klebsiella. aerogenes ppk* genes are followed by a *ppx* gene encoding an exopolyphosphatase [12]. However, no *ppx* homologous gene is found in *Acinetobacter* sp. in the downstream of *ppk* gene, indicating that

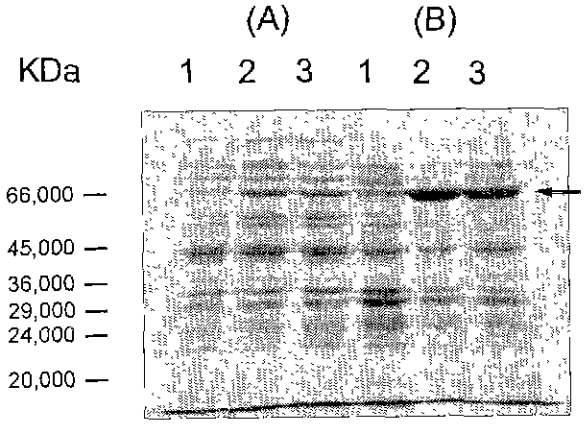

Fig. 4. SDS-polyacrylamide gel electrophoresis patterns showing expression of the *S. marcescens ppk* gene. The thick arrow indicates the position of the band of polyphosphate kinase of *S. marcescens*. Lanes (A) : Soluble fraction, (B) : Insoluble fraction. 1. pkk223-4/JM109, 2. psk10/JM109, 3. psk10/JM109/0.5mM IPTG

the organization of *ppk* and *ppx* in an operon is exception found in *E. coli* and closely related organisms. The proximity and orientation of the genes suggest that the genes encoding the polyphosphate kinase and polyphosphatase may form an operon similar to specific group of bacteria.

Acknowledgement

This work was supported in part by the Korea Research Foundation Fund, in 1998.

References

1. Ahn, K. 1990. Polyphosphate kinase from *Escherichia coli*. *J. Biol. Chem.* **265**, 11734-11739.
2. Akiyama, M., Crooke, E. and Kornberg, A. 1992. The polyphosphate kinase gene of *Escherichia coli*. *J. Biol. Chem.* **267**, 22556-22561.
3. Akiyama M, Crooke, E. and Konberg, A. 1993. An exopolyphosphatase of *Escherichia coli*. The enzyme and its *ppx* gene in a polyphosphate operon. *J. Biol. Chem.* **268(1)**, 633-639.
4. Ara, Z., C. Sudha, and A.M. Chakrabarty. 1999. Cloning and characterization of polyphosphate kinase and exopolyphosphatase genes from *Pseudomonas aeruginosa* 8830. *Appl. Environ. Microbiol.* **65**, 2065-2071.
5. Barford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.
6. Birnbaum, H. C. and Doly. J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7**, 1513-1523.
7. De Crombrugghe, B., Bushy, S., and Buc, H. 1984. Activation of transcription by the cyclic AMP receptor protein. *Science* **224**, 831-838.
8. Gal. S.W., Choi, Y.J. Kim, C.Y. Cheong, Y.H. Choi, Y.J. Bahk, J.D. and Cho. M.J. 1998. Cloning of the 52-kDa chitinase gene from *Serratia marcescens* KCTC 2172 and its proteolytic cleavage into an active 35-kDa enzyme. *FEMS Microbiol. Lett.* **60**, 151-158.
9. Geiofer, W., Ratajczak, A. and Hillen, W. 1998. Tran scription of *ppk* from *Acinetobacter* sp. strain ADP1, encoding a putative polyphosphate kinase, is induced by phosphate starvation. *Appl. Environ. Microbiol.* **64**, 896-901.
10. Hammond, A.E. 1971. Phosphate replacement : problems with the washday miracles. *Science.* **171**, 361-364.
11. Kasahara, M., Makino, K., Amemura, M., Nakata, A. and Shinagawa, H. 1991. Dual regulation of the *ugp* operon by phosphate and carbon starvation at two interspaced promotors. *J. Bacteriol.* **173**, 549-558.
12. Kato, J., Yamamoto, T. Yamada, Yamada, K. and Ohtake, H. 1993. Cloning, sequence and characterization of the polyphosphate kinase-encoding gene(*ppk*) of *Klebsiella aerogenes*. *Gene.* **137**, 237-242.
13. Keasling, J.D., and G.A. Hupf. 1996. Genetic manipulations of polyphosphate metabolism affect cadmium tolerance in *E. coli*. *Appl. Enviro. Microbiol.* **62**, 743-746.
14. Kornberg, A. 1995. Inorganic polyphosphate : toward making a forgotten polymer unforgettable. *J. Bacteriol.* **177**, 491-496.
15. Kuleav, I.S. and V.M. Vagabov. 1983. Polyphosphate metabolism in microorganism. *Adv. Microb. Physiol.* **24**, 83-171.
16. Kumble, K.D., K. Ahn, and A. Kornberg. 1996. Phosphohistidyl active sites in polyphosphate kinase of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **93**, 14391-14395.
17. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature.* **227**, 680-685.
18. Ohtake, H., K. Takashi, Y. Tsuzuki, and K. Tada. 1985. Uptake and relase of phosphate by a pure of *Acinetobacter calcoaceticus*. *Water Res.* **19**, 1587-1594.
19. Ohtake, H., H. Wu, K Imazu, Y. Anbe, J. Kato, and A. Kuroda. 1996. Bacterial phosphonate degradation, phosphate oxidation and polyphosphate accumulation. *Resurces Conservation and Recyling.* **18**, 125-134.
20. Rose, I.A. 1955. Acetate kinase of bacteria(acetokinase). *Methods Enzymol.* **1**, 591-593.
21. Sambrook, J., Fritsch. E F., and Maniatis. T. 1989. Molecular Cloning: a laboratory manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
22. Southern, E.M. 1975. Detection of specitic sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.
23. Wood, H.G. and J.E. Clark. 1988. Biological aspects of inorganic polyphosphates. *Annu. Rev. Biochem.* **57**, 235-260.
24. Yoo, J.S, Kim, H.S. Moon, J.W. Chung, S.Y. and Choi, Y.L. 1998. Analysis and cloning of cAMP receptor protein gene in *Serratia marcescens*. *Kor. J. Life Science.* **8**, 263-271.

초록 : *Serratia marcescens*의 Polyphosphate Kinase 유전자 특성

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본 연구는 인산 축적능이 뛰어난 균주를 분자 육종하여 생물학적 폐수처리 및 토양의 인산 집적을 해결시키는 산업적 유용한 재료로 이용하기 위한 기초연구를 목표로 하고 있다. Polyphosphate kinase는 ATP의 phosphate를 단리하여 한분자씩 결합시키는 형태로 polyphosphate의 합성반응을 촉매한다 인산 축적에 관한 대사과정의 분자적 이해를 위하여 *Serratia marcescens* 균주로부터 Southern hybridization 방법으로 *ppk*를 암호하는 유전자를 찾아내어 재조합시킨 pDH3를 구축 하였다. pDH3으로부터 *ppk*를 암호하는 유전자 영역의 4.0 kb 단편을 가진 subclone을 작성하였다. *Serratia marcescens*의 polyphosphate kinase의 활성은 *catabolite repression*에 의한 조절을 받았다. 발현벡타에 삽입시킨 재조합 플라스미드를 대장균에 도입시킨 결과, polyphosphate kinase의 효소활성이 크게 증가됨을 확인 하였다. 또한 대량 발현시킨 결과를 SDS-PAGE를 통하여 75 KDa의 발현산물을 확인할 수 있었다.