Journal of Life Science 2009 Vol. 19. No. 5. 566~572 ©JLS / ISSN 1225-9918

Molecular Cloning and Analysis of Phosphate Specific Transport (*pst*) Operon from *Serratia marcescens* KCTC 2172

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Received March 4, 2009 / Accepted March 23, 2009

A recombinant plasmid, pDH3, was obtained from the genomic library of *Serattia marcescens* KCTC 2172, and several recombinant subclones constructed from pDH3. The nucleotide sequence of a 5,137 bp segment, pPH4, was determined and three open reading frames were detected. The three ORFs encoded the phosphate specific transport (*pst*) operon, which was *pstC, pstA,* and *pstB*, with the same direction of transcription. Comparison of the *pst* operon of *S. marcescens* with that of other organisms revealed that the genes for *pstS* and *phoU* were missing. A potential CRP bonding site and *pho* box sequence was found in the upstream of the putative promoter at the regulatory region. Analysis of the nucleotide sequence showed that homology in amino acid sequences between the PstC protein and *Yersinia* sp., *Vibrio* sp., and *Pseudomonas* sp. were 49, 37 and 33%, respectively. The PstA protein and *Yersinia* sp., *Vibrio* sp., and *Pseudomonas* sp. showed homologies of 64, 51, and 47%, respectively. PstB protein and *Methanocaldococcus* sp., *E. coli,* and *Mycoplasma* sp. showed homologies of 60, 50, and 48%, respectively. The *pst* genes could be expressed *in vivo* and positively regulated by cAMP-CRP. The *E. coli* strain harboring plasmid pPH7, with *pst* genes, increased with the transport of phosphate.

Key words : *Serratia marcescens*, phosphate specific transport, *pst* operon, pho box

Introduction

Phosphate (P) uptake is of fundamental importance in the cell physiology of because P is required as a nutrient. The P acquisition system is best understood in *Escherichia coli* (*E. coli*), which has evolved several gene clusters allowing the assimilation of P via a variety system. The Pst (phosphate specific transport) system is a high affinity, low-velocity and free-phosphate transport system which is structurally similar to ABC transports. The Pit (phosphate inorganic transport) system is a divalent metal transport for which Pi or arsenate can serve as the anion [12]. A third putative Pi transport gene, *pitB* encodes a functional Pi transporter that may be repressed at low Pi levels by the *pho* regulon [7]. Additionally, several organophosphate transport systems result in Pi uptake in *E. coli*. The *pst* operon consists of five genes, namely, *pstS, pstC, pstA, pstB*, and *phoU*, transcribed anticlockwise on the *E. coli* chromosome. The products of the first four genes are required for the transport of Pi and together with the fifth gene and they are involved in the regulation of the phosphate regulon [3,15,17]. *pstS* from *S. pneumoniae* encodes a phosphate-binding protein; the two following open reading frames (ORFs), *pstC* and *pstA*, encode transmembrane proteins [16] and *pstB* encodes an ATP-binding protein. *phoU*, the terminal gene, has no effect on P_i uptake by the Pst system [6]. The Pst system has also been shown to be functionally conserved in three *Pseudomonas* species for both Pi transport and negative regulation of Pho system [14,20]. Recent studies suggest that the Pst system may modulate the virulence of pathogens, *Edwardsiella tarda* [13]. The *pst* operon of *B. subtilis* has been shown to be induced during alkali stress and analysis of transcriptional regulation [1,2]. Fischer *et al.*, report on the characterization and transcriptional analysis of the *pst* operon at the first gene locus of *C. acetobutyricum* which seems to be a member of a putative Pho regulon [4].

Serratia marcescens is a Gram-negative soil bacterium and a well known chitin decomposer characterized by five types of chitinolytic activities. Several genes, *ChiA* and *ChiB*, have been cloned from *Serratia marcescens* and analyzed [5].

Under ordinary operating conditions, activated sludges are of removing an average of only 20-40 % of the Pi concentrations normally found in municipal wastewaters [9]. The Pi removal capacity of activated sludge appears to be limited by their phosphorous content, which is typically 1 to 2% on

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a dry weight basis [8]. Improving the ability of bacteria to accumulate Pi, therefore, may contribute to excess Pi removal from wastewater. The genetics and understanding of bacterial phosphate transport system is essential for improving their ability to remove phosphate from waste waters [10,19].

This report describes the cloning and analysis of *pst* operon and the demonstration that *pst* genes are Pi transporter. And also describes the genetic improvement of Pi accumulation in *E. coli*.

Materials and Methods

Bacterial strains, plasmid, and culture conditions

Serratia marcescens KCTC 2172 strains were purchased from Korea Culture Type Collection [5]. The host cells were used JM109 and TP2010. pBluscript KS(+), pUC18/19, pHSG398, and pKK223-4 were used for cloning vectors. Bacterial strains were routinely grown at 26°C or 37°C. The antibiotic reagent was used 50 μg/ml ampicillin and 100 μg/ml chloramphenicol. Cultures for enzyme assay of the bacterial cells were grown in minimal medium supplemented with 10 mM potassium acetate and 0.3% casamino acids.

Reagents and chemicals

Restriction endonuclease, klenow fragment, *Taq* DNA polymerase, alkaline phosphatase, and DNA ligation kit were purchased from Takara (Takara Shuzo Co. LTD.). IPTG, X-gal, lysozyme, SDS (Sodium Dodecyl Sulfate), acrylamide, bis-acrylamide, ammonium persulfate, ampicillin, protease K, and RNase were purchased Sigma (Sigma Chemical Co. St. Louis, Mo, USA). Wizard miniprep DNA purification system and wizard genomic DNA isolation kit were purchased from Promega. Agarose and long ranger solution were purchased from FMC (FMC BioProducts, Rockland, ME, USA). All other chemicals were purchased commercially.

DNA isolation and manipulation

Rapid, small-scale plasmid DNA isolation was performed by the Wizard kit of Promega Biotech. All restriction enzymes, T4 DNA ligase, and polynucleotide kinase were used according to the recommendation of the suppliers. Recombinant DNA techniques were performed by the method of Sambrook and Russel [18].

Sequencing and analysis

Determination of DNA sequences was performed by the dideoxynucleotide chain termination method by on Autocycle DNA sequencing kit using ALF express DNA sequencer (Pharmacia). DNA was subcloned in pUC18, pUC19, and pBluescript KS(+) using restriction fragments of the cloned plasmid. Other subcloned fragments for sequencing were constructed by the deletion method using exonucleaseIII-Mung Bean nuclease-klenow enzyme kit (Takara Co LTD, Japan) after subcloning of the inserted fragment and cloned into pBluescript KS(+).

Phosphate uptake experiment

Cells were cultured in LB medium at 37°C with shaking for overnight. The cultured cells were transferred into P1 medium at optical density about 0.2 at A_{600nm}. The cell suspension was incubated at 26°C with constant shaking and samples were taken at intervals for determination of growth and Pi concentration. Pi uptake by *E. coli* containing recombinant plasmids were determined by Pi removal from the medium. The Pi-concentration was estimated by the method of ammonium molybdate using Sigma Diagnostic Kit 360-UV (Sigma, St. Louis. Mo). Cell growth was determined by measuring OD_{600}.

β-galactosidase assay

The regulation of expression of the *pst* gene in *E. coli* was investigated by a gene fusion technique. To construct a plasmid encoding a *pst-lac* fused protein, the 600 bp PCR fragment of the *pst* regulatory region was obtained from pDH3 and ligated with *Sma*I digested *LacZ* fusion vector pMC1403. TP2010 (*ΔLac*, *Δcya*) was transformed by the ligation mixture and the transformants were selected on the MacConkey-lactose plates containing 50 μg/ml ampicillin. The red colonies were picked up and the plasmids in the cells were purified. The strain TP2010 containing *pst-lac* fusion gene was incubated in M9 medium at 37°C until the OD_{610nm} reached 0.1-0.2, and the 0 or 1 mM cAMP was added to each sample. Each sample was further incubated until the OD_{610nm} was 0.5 and the β-galactosidase activity was determined as described previously [21]. All assays were carried out in triplicate and the data represent means of three replicates.

Nucleotide sequence accession number

The nucleotide sequences of the phosphate specific transport (*pstCAB*) operon reported in this article have been assigned GenBank accession number AF280069.

Results and Discussion

Cloning of the phosphate transport gene from *S. marcescens KCTC 2172*

In a previous study, the pDH3, recombinant plasmid DNA, was obtained from *S. marcescens* genome library, which contained *ppk, ppx,* and *pst* operons [10,11]. Several recombinant subclones were constructed from pDH3. One of the subclones, pSH4, was further analyzed. In order to investigate the increased expression of the gene under the direction of phosphate transport, a restriction enzyme map of the pSH4 was constructed (Fig. 1). On analyzing the nucleotide sequence of the partial region of pSH4 with a gene analysis data bank, three open reading frames (ORFs) were observed. The ORFs were homologous to several bacteria genes encoding phosphate transport genes (*pstC, pstA*, and *pstB*). In the case of *S. marcescens*, there are three ORFs presumably forming an operon and following the same direction of transcription. The first ORF, *pstC* was contained a partial *pstC* gene in pSH4. For cloning of the *pst* operon region containing the complete *pstC* gene, the *Sph*I-*Hin*dIII fragment (about 5 kb) of pDH3 cloned into *Sph*I-*Hin*dIII digested pHSG398 to construct pPH4. To obtain the *pstC, pstA*, region of *S. marcescens*, the *Sph*I-*Eco*RV fragment of pDH3 cloned into *Sph*I-*Sma*I digested pUC19 to construct pSAC5 and to obtain the only *pstB* region of *S. marcescens*, two PCR primer were designed SerB1: 5′-GCATCTCGACATGAACA GACCGCGCTGG-3′ and SerB2: 5′-GTATGGGTGTGCCCGT TTGGGCTATGAA-3′. These primers were created 800 bp, using recombinant pDH3 as a template. This PCR product was cloned into a pT7 vector to construct pSB5. The restriction map of the cloned DNA fragments and several recombinant subcloned genes were constructed.

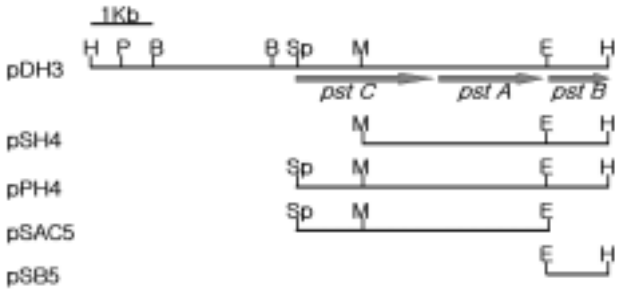


Fig. 1. Physical map of recombinant plasmid DNA, pDH3, from *S. marcescens* KCTC 2172 and derivative plasmids. The initiation site and direction of transcription of *pst* genes are shown. Abbreviations: B, *Bam*HI; P, *Pst*I; E, *Eco*RI; H, *Hin*dIII; S, *Sph*I.

Structural analysis of phosphate transport genes

For the recombinant plasmid DNA, pPH4, consisted of 5,137 bp (Fig. 2). The nucleotide and deduced amino acids sequences were analyzed using FASTDB and BLAST. These fragments encoded three open reading frames (ORFs), a *pst* (phosphate specific transport) operon. The *pst* genes constituted *pstC, pstA,* and *pstB*, and were oriented in the same direction of transcription. The putative promoter region with -10 box (TATCTT) and -35 box (CTGACA) was spaced by 18 nucleotides. In comparison with the consensus promoter sequence, the regulatory region has a highly conserved -10 box and -35 box. Potential *pho* box sequence was found in upstream of the putative promoter in the regulatory region. There was a 14/18 bp match with the consensus *pho* box sequence. The *pho* box-binding protein of *S. marcescens* will recognize their sequence and regulate the transcription of this operon. The consensus sequences of the *E. coli* CRP binding site were found upstream of 190 bp of a start codon. Sequencing matching the consensus 5′-AANT GTGANNTA NNTCACATTT-3′ of the *E. coli* CRP binding site was found at upstream of the putative promoter region. There was an 11/17 bp match between the consensus CRP binding sequence and *Serratia* sp. binding site 5′-AAGCGT GTTTACGTTCATTTTT-3′. Therefore, we expect that the expression of *pst* genes could be regulated by the cAMP-CRP complex. The *pst* genes in *E. coli* [3] and *C. acetobutylicum* [4] form an operon arranged in the order *pstS, pstC, pstA, pstB,* and *phoU*. In the case of *B. subtilius* is missed the *phoU* [17] and *P. aeruginosa* [15] is missed the *pstS*. Comparison of the *pst* operon from *S. marcescens* with other organisms revealed that the gene corresponding to *pst*S and *pho*U is missed (Fig. 3). Thus, the gene architecture of the *pst* operon of *S. marcescens KCTC2172* is identical to that of *P. aeruginosa* but interestingly quite different from the more closely related *E. coli*. Each ORF was preceded by a putative ribosome binding site appropriately spaced from a presumed ATG initiation codon. The *pstC* gene containing a putative ATG start codon at nucleotide positions 496 and a stop codon at positions 2,527. The predicted gene product of *pstC* was composed of 678 amino acids residues with molecular mass 74 kDa. Analysis of the nucleotide sequence showed that homology in amino acid sequence between PstC protein and *Yersinia pestis*, *Vibrio cholerae*, and *Pseudomonas aeruginosa* was 49, 37, and 33%, respectively. The *pstA* gene of 1,478 bp started at position 2,629 and proposed an initiator codon for *pstA*. The *pstA* gene could code for a protein of

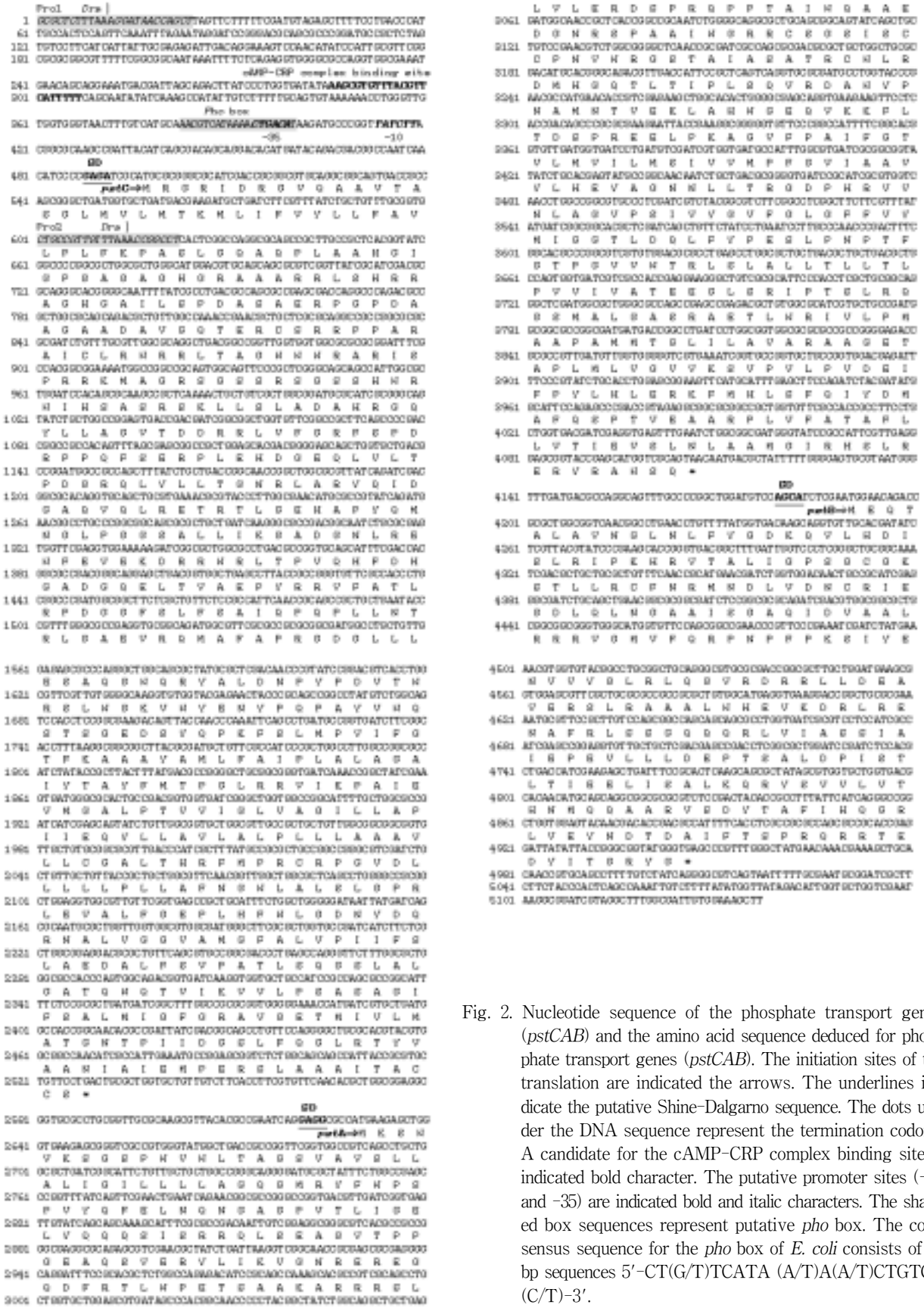


Fig. 2. Nucleotide sequence of the phosphate transport genes (*pstCAB*) and the amino acid sequence deduced for phosphate transport genes (*pstCAB*). The initiation sites of the translation are indicated the arrows. The underlines indicate the putative Shine-Dalgarno sequence. The dots under the DNA sequence represent the termination codons. A candidate for the cAMP-CRP complex binding site is indicated bold character. The putative promoter sites (-10 and -35) are indicated bold and italic characters. The shaded box sequences represent putative *pho* box. The consensus sequence for the *pho* box of *E. coli* consists of 18 bp sequences 5′-CT(G/T)TCATA (A/T)A(A/T)CTGTCA (C/T)-3′.

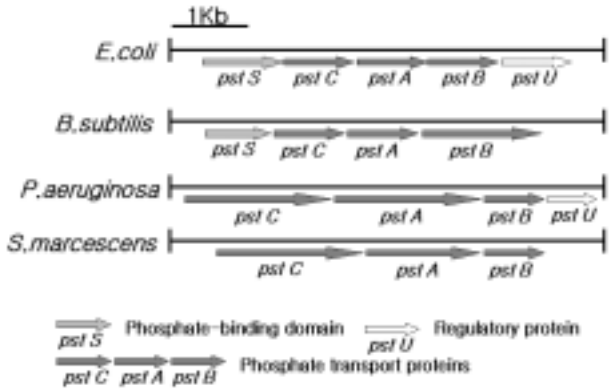


Fig. 3. Comparison of the structural organization of the *pst* operon of *Serratia marcescens* KCTC 2172 with that of other organisms. Sizes of ORFs are to arrow scale.

516 amino acid residues having a calculated 55 kDa. Analysis of the nucleotide sequence showed that homology in amino acid sequence between the PstA protein and *Yersinia* sp., *Vibrio* sp., and *Pseudomonas* sp. was 64, 51, and 47%, respectively. The *pstB* gene of 758 bp started at position 4,189. The *pstB* gene could code for a protein of 253 amino acid residues with molecular mass of 25 kDa. Analysis of the nucleotide sequence showed that homology in amino acid sequence between the PstB protein and *Methanococcus* sp., *E. coli*, and *Mycoplasma* sp. was 60, 50, and 48%, respectively (data not shown).

Expression of the *pst* gene of *S. marcescens* KCTC 2172 in *E. coli*

Genes for the putative *pst* operon region of *S. marcescens* was introduced into the bacterial expression vector pHSG398. The putative *pst* operon region of pDH3 was digested with the *Sph*I-*Hin*dIII (about 5 kb) and cloned into the same site of pHSG398 to construct pPH4. Regulation of expression of the *pst* operon in *E. coli* was investigated with a *pst-lacZ* fusion plasmid. To construct the plasmid encoding a *pst-lacZ* fusion protein, a PCR product of DNA fragment of about 600 bp was amplified with two primers (Pro1: 5′-AGGCCGGTTTGAACAACGGCAG-3′ and Pro2: 5′-AGC TGGTTATCCTTTAATGAGCGC 3′). This 600 bp, from the *Dra*I to the *Dra*I fragment of the pDH3 plasmid, contains the *pst* regulatory region including codons of the *pst* gene. It was inserted in the *Sma*I site of the plasmid pMC1403 to construct the recombinant plasmid pMR2. The nucleotide the sequence of the pMR2 and pattern of the restriction enzyme indicated that the reading frames of the *pst* and *lacZ* were located in the same direction. After induction of *pst* genes with 0.5 mM IPTG, cell extracts were prepared from cultured bacterial cells. To examine whether the expression of the *pst* gene was regulated by a cAMP-mediated process, the β-galactosidase activity was measured at various times after the addition of cAMP to the medium in which the *E. coli* cells contained plasmid pMR2 (Table 1). When cAMP was added, the β-galactosidase activity increased more than without cAMP. Expression of the gene encoding the fused protein was increased by the cAMP-CRP complex. These results showed that the *pst* gene could be expressed *in vivo* and positively regulated by cAMP-CRP.

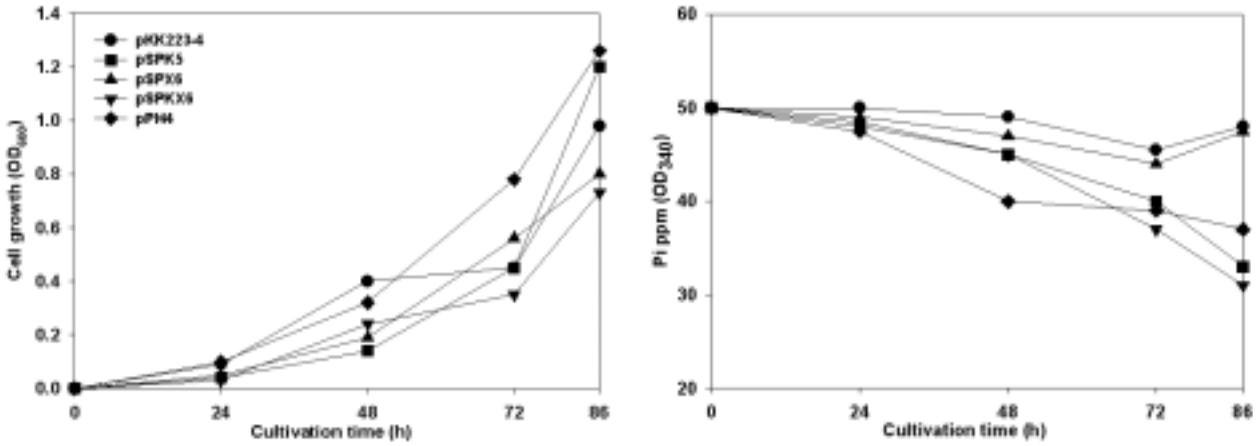
Phosphate removal by the recombinant plasmids in *E. coli*

The cell suspension was incubated at 26°C with constant shaking. Samples were taken at intervals to determine the growth and Pi concentration. Pi uptake by *E. coli* recombinant plasmids were determined by Pi removal from the medium. Pi uptake experiments were performed in duplicate with strain *E. coli* containing pKK223-4, a control strain, and four recombinant derivatives. Fig. 4 illustrates the growth and Pi uptake by strain *E. coli* containing pKK223-4, and recombinant derivatives containing pSPK5 with *ppk* region, pSPX6 with *ppx* region, pSPKX6 with *ppk* and *ppx* regions and pPH4 with *pst* operon. Growth of the four recombinant strains was almost equivalent to that of the control strain. The control strain removed about 10% of the Pi from the medium during the first 4 days. However, no Pi uptake occurred after growth stopped and the cell growth of strain decreased after reaching a maximum at 4 days (data not shown). The decrease in cell density was concomitant with an increase in Pi in the medium, indicating that Pi was released by cell lysis. Strain *E. coli*, containing pSPK5 and pSPKX6, removed about 40%, four-fold more Pi from the medium than did the control strain. Presumably, the *E. coli* recombinant strain containing pSPK5 alone removed a little

Table 1. Effect of cAMP on the expression of the *pst-lacZ* fusion proteins

Strain	Plasmids	β-galactosidase activity[a]			
		-cAMP	+cAMP	Effect[b]	Index[c]
E. coli TP2010	pMC1403	68	72	1.06	100
E. coli TP2010	pMR2	1246	2451	1.97	185

[a]: β-galactosidase activities are given in Miller Unites. [b]: Relative efficiency with that in the absence of cAMP taken as 1. [c]: Relative efficiency with that in the wild type taken as 100. Data represent the means of three replicates.



(a) (b)

Fig. 4. Time course of cell growth (a) Pi concentration (b) during growth of *E. coli* JM109 containing pKK223-4, pSPK5, pSPX6, pSPKX6, and pPH4 in P1 medium containing 50 ppm Pi. Data represent the means of three replicates.

more Pi from the medium than did strain *E. coli* containing pSPKX6. The strain *E. coli* containing pSPX6 and pPH4 took up a little Pi, even compared with the control strain. The strain *E. coli* containing pPH4 alone could not remove more Pi than did the strain *E. coli* containing pSPK5 and pSPKX6. Therefore, further work would be needed to construct *E. coli* recombinant strain containing *ppk* region and *pst* operon. That *E. coli* recombinant strain presumably would remove more Pi. These results indicate the potential for genetic improvement of more useful microorganisms that enhance Pi removal.

Acknowledgement

This paper was supported by the Dong-A University Research Foundation.

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초록 : *Serratia marcescens* KCTC 2172로부터 pst operon의 클로닝 및 해석

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S. marcescens KCTC 2172로부터 유전자 은행을 작성하여 재조합 클론 pDH3를 얻었으며, pDH3 유래의 서브클론을 작성하였다. 플라스미드 pPH4의 전염기서열 5,137 bp 영역을 결정한 결과 3개의 ORF가 있음을 확인하였다. 이들은 *pst* 오페론의 *pstC, pstA,* 및 *pstB*, 세 유전자를 동일 전사방향으로 코드하고 있었다. 타 세균의 유전자와 비교한 결과 *S. marcescens*의 *pst* 오페론은 *pstS*와 *phoU*가 결손되어 있다. 조절영역에는 CRP 결합영역과 *pho* box 서열이 존재하였다. 보고된 유전자와 상동성 조사결과, PstC 단백질은 *Yersinia* sp., *Vibrio* sp. 및 *Pseudomonas* sp.와는 49, 37, 33%의 상동성을, PstA 단백질은 *Yersinia* sp., *Vibrio* sp. 및 *Pseudomonas* sp.와 64, 51, 47%의 상동성을, PstB 단백질은 *Methanocaldococcus* sp., *E. coli* 및 *Mycoplasma* sp.와 60, 50, 48%의 상동성을 나타내었다. *Pst* 유전자들은 조절영역의 cAMP-CRP 복합체에 의해 *in vivo*에서 양성적으로 발현됨을 확인하였다. Pst 오페론을 포함하는 플라스미드를 도입한 대장균은 인산운송에 관여하는 능력을 확인하였다.