J. Microbiol. Biotechnol. (2006), **16**(8), 1285–1291

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Regulatory Characteristics of the *Vibrio vulnificus putAP* Operon Encoding Proline Dehydrogenase and Proline Permease

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Received: April 1, 2006
Accepted: April 26, 2006

Abstract The proline utilization (*put*) operon of *Vibrio vulnificus* consists of the *putAP* genes encoding a proline dehydrogenase and proline permease. The result of *put-lux* transcriptional fusion analysis suggests that the *V. vulnificus putAP* operon is not autoregulated by the PutA protein. A *putR* null mutation decreased proline dehydrogenase activity and the level of the *put* transcripts, indicating that transcription of *putAP* is under the positive control of PutR. The deduced amino acid sequence of the *putR* was similar to those reported from other bacteria with high levels of identity. Chromatin IP and GST pull-down assays revealed that PutR specifically binds to the *putAP* promoter region *in vivo*, and interacts with CRP *in vitro*. Taken together, the results suggested that PutR exerts its effect on *putAP* expression by directly interacting with CRP bound to the upstream region of P_{put}.

Key words: *Vibrio vulnificus*, *putAP*, PutR

Like many other foodborne pathogenic bacteria, *Vibrio vulnificus* occurs in various environments having different osmotic strengths; it naturally inhabits coastal seawaters, contaminates shellfishes, survives the present control practices such as adding salt or sugar to suppress its growth, and colonizes in the human body. This indicates that *V. vulnificus* has to cope with everchanging osmolarity in its growth environments. Previously, we had cloned the *putAP* genes encoding a proline dehydrogenase and a proline permease of *V. vulnificus* (Fig. 1) [12]. Functions of the *putAP* genes were assessed by the construction of mutants, and the gene products of *putAP* appeared to contribute to the osmotic tolerance of *V. vulnificus* [12, 13].

In enteric bacteria, the *putA* gene belongs to the *put* operon together with the divergently transcribed *putP* gene.

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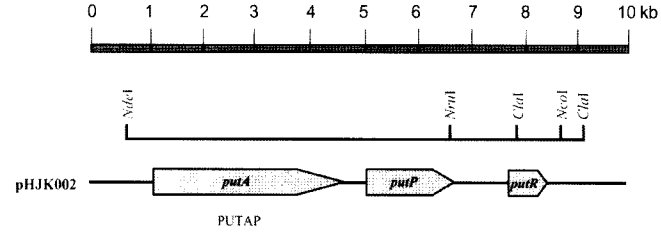

Fig. 1. Schematic representation of *V. vulnificus put* genes cloned in pHJK002.
The arrows represent the transcriptional directions and coding regions of the *put* genes. The DNA probe, PUTAP, used for Northern blot analyses is depicted by a closed bar.

In *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Klebsiella pneumoniae*, in addition to its enzymatic activity, the PutA protein also functions as an autogenous transcriptional repressor of the *putA* and *putP* genes [1, 16, 17, 19, 21]. However, the amino acid sequence homology of *V. vulnificus* PutA with those of *E. coli* and *S. typhimurium* is quite lower than that observed between PutA's from *E. coli* and *S. typhimurium*. Furthermore, the organization of the *putAP* genes of *V. vulnificus* is different from that of other enteric bacteria, such that the transcription orientations of both genes are the same direction rather than divergent [13].

It has been demonstrated that the *putAP* genes of *V. vulnificus* are expressed and modulated by a way different from that of the enteric bacteria. Expression of the *V. vulnificus putAP* genes is induced by hyperosmotic stresses and activated by CRP and PutR [13, 14]. Here, to extend our understanding of the regulation of the *putAP* genes, we determined whether *V. vulnificus putAP* genes are autoregulated by PutA as observed in the enteric bacteria. The nucleotide and deduced amino acid sequences of the *putR* gene were analyzed and the possible role of the PutR protein in regulation of the *putAP* expression was explored.

Table 1. Plasmids and bacterial strains used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
V. vulnificus		
ATCC29307	Clinical isolate; virulent	Laboratory collection
HJK001	ATCC29307 with *putA::nptI*; Km^r	[12]
HJK003	ATCC29307 with *putR::nptI*; Km^r	[14]
E. coli		
DH5α	*supE44Δ lacU169 (ϕ80 lacZ* ΔM15*) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
Plasmids		
GST-CRP	pGEX-4T-1::*crp*; Ap^r	This study
pGEX-4T-1	GST fusion vector; Ap^r	Amersham
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	[10]
pHJK002	pUC18 with *putAPR*; Ap^r	[12]
pHJK0069	pRK415 with *putR*; Tc^r	[14]
pHK0011	pRK415 with promoterless *luxAB*; Tc^r	[7]
pJH0201	pHK0011 with 750-bp fragment of *putAP* upstream region; Tc^r	This study

[a]Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Media

The strains and plasmids used in this study are listed in Table 1. The *E. coli* strains used for the plasmid DNA replication or conjugational transfer of the plasmids were grown in a Luria-Bertani (LB) broth with or without 1.5% (w/v) agar. Unless otherwise noted, the *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). When appropriate, antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml. M9-P was a medified M9, in which 100 mM proline was supplemented and buffered to pH 7.0 with 100 mM *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid [TES].

General Genetic Methods

The procedures for the isolation of plasmid DNA, genomic DNA, and transformation were carried out as described by Sambrook and Russell [22]. The restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs Inc., Beverly, MA, U.S.A.). The DNA fragments were purified from agarose gels using a High Pure PCR product purification kit (Roche, Mannheim, Germany). The primary DNA cloning and manipulation were conducted in *E. coli* DH5α, and restriction mapping was used to confirm that transformants contained the appropriate plasmids. PCR amplification of DNA was performed using a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, U.S.A.) following standard protocols.

Measurement of Proline Dehydrogenase Activity

Cultures of *V. vulnificus* strains in M9-P broth were grown at 30°C with aeration. Samples of 5 ml were removed at OD_{600} 0.8 for determination of cell densities, proline dehydrogenase activities, and level of *put* transcripts. The proline dehydrogenase activities were determined as previously described [12]. A unit of the enzyme activity was defined by the method of Ostrovsky *et al.* [20]. Protein concentrations were determined by the method of Bradford [2], with bovine serum albumin as the standard. Averages and standard errors of the mean (SEM) were calculated from at least three independent trials.

Transcript Analysis

The total cellular RNA was isolated from the *V. vulnificus* strains using a Trizol reagent kit (Invitrogen, Carlsbad, CA, U.S.A.). For Northern blot analysis, a series of reactions was performed according to standard procedures [22] with 20 μg of total RNA. RNA was transferred to a nylon membrane, and hybridized as previously described [8]. A 960-bp DNA fragment, containing the coding region of *putA*, was amplified by PCR using primers PUTA004 (5'-CATGAATTCCTCCTTATGCGTTCTCA-GTC-3') and PUTA005 (5'-ACTCTAGATTCGGGGAT GAGATTGAGGAAA-3') and then labeled with [α-^{32}P]dCTP using the Prime-a-gene labeling system (Promega, Madison, WI, U.S.A.) and named PUTAP (Fig. 1). The blots were visualized and quantified using a phosporimage analyzer (Model BAS1500, Fuji Photo Film Co. Ltd, Tokyo, Japan) and the Image Gauge (version 3.12) program.

Construction of a *put-luxAB* Transcriptional Fusion

A *put-luxAB* transcriptional fusion reporter was created by subcloning of a DNA fragment that overlapped the *putAP* promoter region into pHK0011 (Fig. 2A) that was carrying promoterless *luxAB* luciferase genes [7]. The 750-base pair upstream regulatory region of the *putAP* from pHJK002

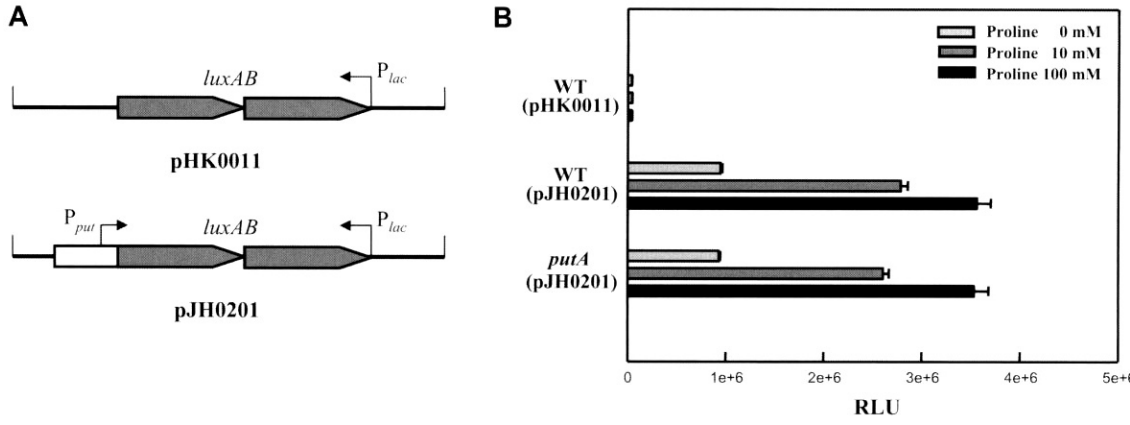

Fig. 2. Proline-responsive induction of a *put-luxAB* transcriptional fusion in wild-type and *putA* mutant.
A. Construction of *put-lux* fusion plasmid pJH0201. *Filled blocks*, the *luxAB* DNA; *open block*, the *putAP* regulatory region; *solid lines*, the vector DNA used. **B**. Cellular luminescences determined from the wild-type and isogenic *putA* mutant of *V. vulnificus* containing the *put-lux* reporter. Strains were cultured in M9 medium supplemented with the indicated concentrations of proline. Cultures in log phase of growth were used to measure cellular luminescence. Error bars represent the SEM.

[12] was amplified by PCR using the two primers PUTA0010 (5'-TCGGGTACCATCTCACCTTTATTTTCA-3') and PUTA0011 (5'-TTAGGATCCATCAATGCCC AT-AGCTTA-3') and inserted into pHK0011 digested with BamHI and KpnI to yield pJH0201 (Fig. 2). pJH0201 was transferred into ATCC29307 and the isogenic *putA* mutant by conjugation. The cellular luminescence of the cultures was measured with a luminometer (Lumat Model 9507, Berthold, Wildbad, Germany) and expressed in arbitrary relative light units (RLU) as described previously [5, 9].

Western Blot Analysis of *V. vulnificus* PutR

The purified His-tagged PutR was used to raise polyclonal antibodies to the PutR of *V. vulnificus* as previously described [14]. For the Western immunoblotting, the proteins were resolved by SDS-PAGE [22]. The resolved proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.) and probed with a 1:5,000 dilution of the rat polyclonal antibodies (Takara, Seoul, Korea). The bound antibodies were detected using goat anti-rat IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO, U.S.A.), and visualized by incubation with a 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate (Sigma) [7].

Chromatin Immunoprecipitation

The chromatin immunoprecipitation experiments were performed using formaldehyde cross-linking as described by Spencer *et al.* [24] with minor modifications. The wild-type and HJK003 were grown to an OD_{600} of 0.8, and the cross-linking proceeded at 20°C for 20 min, by adding formaldehyde (final concentration of 1%) directly to 50 ml of the cultures. After stopping the cross-linking with the addition of glycine to a final concentration of 0.125 M, the cross-linked cells were harvested, washed, and finally resuspended in 1 ml PBS (pH 7.4). The cross-linked chromatin in the cells was fragmented by sonication to result in sheared chromatins with average lengths of approximately 500-bp.

One-half of the clarified supernatant was saved as the total input sheared chromatin (positive control) prior to the reaction with the anti-PutR antibody. The sheared chromatin (100 μl) from the other half of the supernatant was reacted with 10 μl of the anti-PutR antibody overnight at 4°C, and the resulting chromatin-antibody complex was specifically precipitated by adding 45 μl of 50% protein A-Sepharose (Amersham). The precipitates were washed and the sheared chromatins were eluted using methods mentioned elsewhere [24]. The cross-linkings were reversed by incubating the sheared chromatins with 1% SDS and 0.1 M $NaHCO_3$ at 65°C for 6 h, and DNAs were purified and analyzed by a PCR using the primers PUTA0313 (5'-CCACTCCTTTACT CGCTTACAG-3') and PUTA0314 (5'-AACTCCGGCTTTAACACATCT-3').

Purification of GST-CRP Fusion and GST Pull-Down Assay

The *crp* coding region was subcloned into a glutathione *S*-transferase (GST) gene fusion vector, pGEX-4T-1 (Amersham, Buckinghamshire, U.K.), to result in GST-CRP (Table 1). The GST-CRP fusion protein was then expressed in *E. coli* BL21 (DE3), and purified by glutathione-sepharose 4B beads according to the manufacturer's procedure (Amersham).

For GST pull-down assays, GST-CRP was immobilized on glutathione-sepharose 4B beads (Amersham) and incubated with His-tagged PutR at 4°C for 1 h in incubation buffer (10 mM Tris-Cl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.1 mg/μl BSA) [23]. In a similar fashion, purified GST were bound to beads for use as a negative control. The CRP and PutR binding was conducted without and with

cAMP (1 mM). After extensive washing with PBS, proteins were eluted by boiling in SDS gel-loading buffer [22] and resolved in 12% SDS-PAGE, and then PutR was detected by Western blot analysis using anti-PutR antibody.

RESULTS

Expression of *V. vulnificus putAP* is Not Autoregulated by PutA

It has been suggested that in *E. coli* and *S. typhimurium*, the PutA protein represses its own transcription. To determine whether the *V. vulnificus putAP* is autoregulated by PutA, we assessed the expression of the *putAP* promoter P_{put} by constructing a *put-lux* fusion reporter plasmid, pJH0201 (Fig. 2A). The parent plasmid pHK0011, carrying a promoterless *luxAB*, was used as a control. Strains were cultured to log phase in M9 medium supplemented with proline as indicated (Fig. 2B) and the levels of luminescence were determined. The levels of luminescence increased in the presence of proline, supporting our previous observation that P_{put} is induced by proline [13]. However, the luminescence level of the *putA* mutant cells was not significantly different from that of the wild-type. This result indicated that the activity the P_{put} was not repressed by PutA. This result suggests that the *V. vulnificus putAP* operon is transcriptionally induced by exogenous proline rather than autoregulated by the PutA protein.

Amino Acid Sequence Analysis of *V. vulnificus* PutR

In a previous study, we identified and cloned *V. vulnificus putAP* genes by a transposon-tagging method [12]. An interesting candidate for the regulatory gene was found by sequencing the downstream region of *putAP* (Fig. 1, 14). The nucleotide sequences of the ORF in pHJK002 were determined by primer walking (Korea Basic Science Research Center, Gwangju, Korea). The nucleotide sequence revealed a coding region consisting of 495 nucleotides (Fig. 1). The amino acid sequence deduced from the *putR* coding sequence revealed a protein, composed of 164 amino acids with a theoretical molecular mass of 18,709 Da and a pI of 6.44. A database search for amino acid sequences similar to those deduced from the coding regions revealed other PutR (proline utilization regulator) proteins from *Agrobacterium tumefaciens* and *Rhodobacter capsulatus* [4, 11], with high levels of identity.

This information proposed that the coding region is a homolog of the *putR* genes reported from other bacteria, and led us to name the coding region as *putR* of *V. vulnificus*. The amino acid sequence of the *V. vulnificus* PutR was 87% identical to the putative transcriptional regulator of *Vibrio cholerae*, which is located downstream of *putAP*, 47% identical to the PutR protein of *A. tumefaciens*, 46% identical to the Lrp protein of *E. coli* (Fig. 3), and is similar to other members of the Lrp family of transcriptional regulators (data not shown). These regulatory proteins are characterized by a DNA-binding helix-turn-helix motif in the N-terminus [6], which is also highly conserved in *V. vulnificus* PutR (Fig. 3).

Effect of *putR* Mutation on the Expression of *putA* and *putAP* Transcripts

The high level of identity found in the amino acid sequences for the PutR protein from *V. vulnificus* and other bacteria indicated that they might perform a similar function in their physiological roles. To examine whether the expression of *V. vulnificus putAP* is activated by PutR, the proline dehydrogenase activity of an isogenic *putR* mutant, in which chromosomal *putR* is disrupted by insertion of an *nptI* gene [14], was compared with that of the wild-type. When the *putR* mutant HJK003 was compared with its parental wild-type during the log phase, the level of proline dehydrogenase activity was 2-fold less than in the wild-type strain (Fig. 4A). This result suggested that PutR positively regulates *putA* gene expression, as observed previously [14].

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                                                  H-T-H
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V. vulnificus      MSD------YPLDRIDLQILRILHLKGRLPVVELAKQVNLTTSPCSERVKRLEKEGYIKG 54
V. cholerae        MS---------LDRVDRDILRILHAKGRLPVVELAKLVNLTTSPCSERVKRLEKEGYIRG 51
A. tumefaciens     MANSHKT--DDLDHFDLKILEALSEDGRMSVLQLSKRVGLSKTPCQTRLKRLVDEGYILG 58
E. coli Lrp        MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVRRLERQGFIQG 60

V. vulnificus      YHAELNAEKLGLDVQVFIHIRLDQTSFSIFEKFAKAVADMPEIEECYSLSGDFDTMIKVR 114
V. cholerae        YHADLDPGKLGLDVQVFIHIRLDQSSFSIFERFAHAVADIPEIEACYSLSGDFDTMIKVR 111
A. tumefaciens     FRAVLNPQKLGVDHIAFAEVKLSDTREKALEEFNTAVRKIKEVEECHMIAGAFDYLLKVR 118
E. coli Lrp        YTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNTAVQKLEEIQECHLVSGDFDYLLKTR 120

V. vulnificus      VKSMKAYQEFMSSKLGTLPGVIQTRSEVVIEEHKTGFGVNPELLSTLYQK 164
V. cholerae        VKDMKAYQAFMSGKLGSLPGVIQTRSEFVIEEHKTSFGINPELIYSLPS- 160
A. tumefaciens     TSDIRKYRRVLGEKISSLPSVSNTSTFVVMQSVK---ETG---I------ 156
E. coli Lrp        VPDMSAYRKLLGETLLRLPGVNDTRTYVVMEEVK---QSNRLVIKTR--- 164
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Fig. 3. Alignment of the PutR amino acid sequence with those of other bacteria.

Residues in gray shading are identical in all members, and dashes represent missing sequences. Alignment was based on the amino acid sequences in the GenBank (NCBI) database and derived by the CLUSTALW alignment program (http//www.ebi.ac.uk/clustalw). A putative DNA-binding helix-turn-helix motif (H-T-H) is marked.

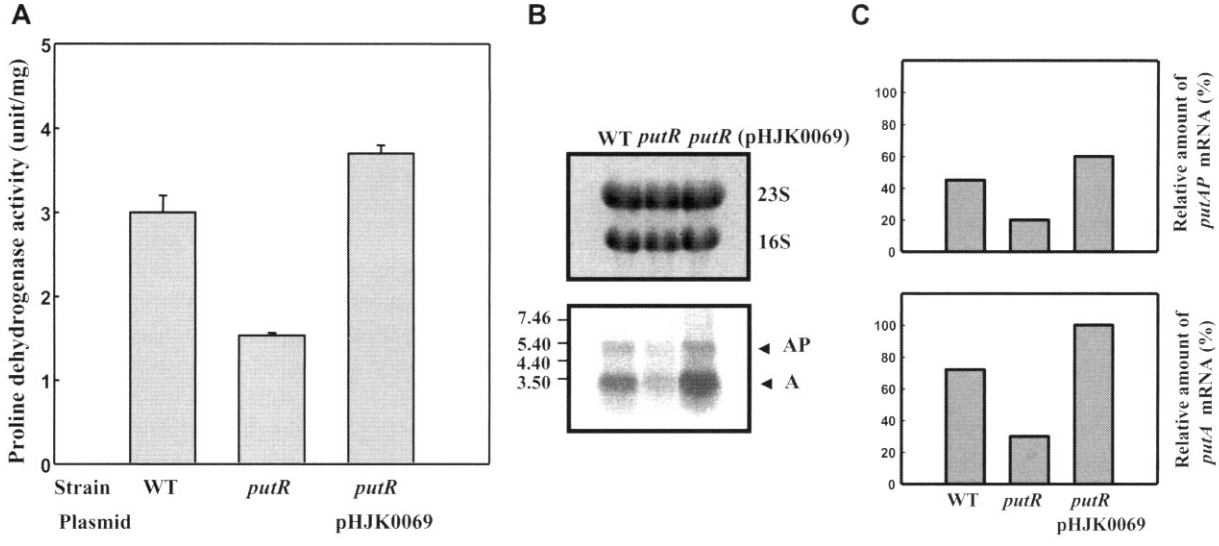

Fig. 4. Dependency of proline dehydrogenase production of *V. vulnificus* on *putR*.
Proline dehydrogenase activities (**A**), and relative amounts of the *put* transcripts (**B** and **C**), were determined for the wild-type strain and the isogenic *putR* mutant, HJK003, as indicated. Complementation of the *putR* mutation by functional *putR* (pHJK0069) is also presented. Samples removed from each culture grown to the log phase in LBS were analyzed for proline dehydrogenase activity and *put* transcripts. Error bars represent the SEM. The relative amounts of the *put* transcripts in each band are expressed relative to the amount of the transcript A of HJK003 (pHJK0069).

To characterize the role of PutR in more detail, the levels of *put* transcripts in the wild-type strain and HJK003 were compared by Northern blot analysis. It has been revealed that transcription of *putAP* genes results in two different transcripts, transcript A (*putA* transcript) and transcript AP (*putAP* transcript) [13]. When PUTAP was used as the DNA probe, the level of *put* transcripts in the *putR* mutant was reduced lower levels than that observed in the wild-type strain (Figs. 4B and 4C). These results indicated that PutR exerted its effects on the production of proline dehydrogenase at the level of transcription of both *put* transcripts. We examined whether the reintroduction of recombinant *putR* could complement the decrease in proline dehydrogenase activity in HJK003. For this purpose, plasmid pHJK0069 was constructed by subcloning the *putR* coding region, which was amplified by PCR, as described elsewhere [14], into pRK415 under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter [10]. When *putR* was induced by IPTG, the proline dehydrogenase activity and *put* transcripts of HJK003 (pHJK0069) in the log phase were restored to levels comparable to those in the wild-type (Fig. 4).

PutR Binding to Upstream Region of *putAP In Vivo*

Although the gel-mobility shift assays with purified PutR revealed that PutR binds to the *putAP* promoter region [14], there was still no proof that the same binding of PutR to the *putAP* promoter DNA also occurs in *V. vulnificus*. Thus, to determine whether PutR binds to the *putAP* promoter *in vivo*, the cross-linked chromatin from the wild-type and *putR* mutant HJK003 cells was immunoprecipitated using the antibody against PutR (Fig. 5). As positive controls, the input chromatin from both the wild-type and HJK003 appeared to carry the *putAP* promoter DNA (Fig. 5). After reversing the cross-links, the *putAP* promoter fragment was detected in the chromatin precipitate from the wild-type that is induced with the anti-PutR antibody. The primers were designed to specifically amplify the *putAP* promoter DNA that is 286-bp in length. The presence of the *putAP* promoter DNA in the precipitated chromatin was caused by the specific binding of the PutR protein to the DNA, since no *putAP* promoter DNA was detected in the precipitate induced in the absence of the anti-PutR

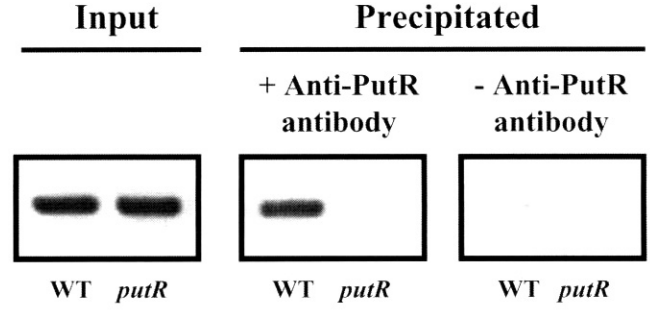

Fig. 5. PutR directly binds to the *putAP* promoter *in vivo*.
Wild-type and isogenic *putR* mutant of *V. vulnificus* were grown to the log phase. The cells were cross-linked with formaldehyde, washed, and then sonicated to produce sheared chromatin, as described in the Materials and Methods. The DNA was purified from the sheared chromatins before being precipitated (Input, positive control) and after precipitation with the protein A-Sepharose in the presence (+) or absence (−) of anti-PutR antibody. The DNA was then amplified by a PCR using primers specific to the *putAP* promoter. WT, wild-type; *putR*, *putR* mutant.

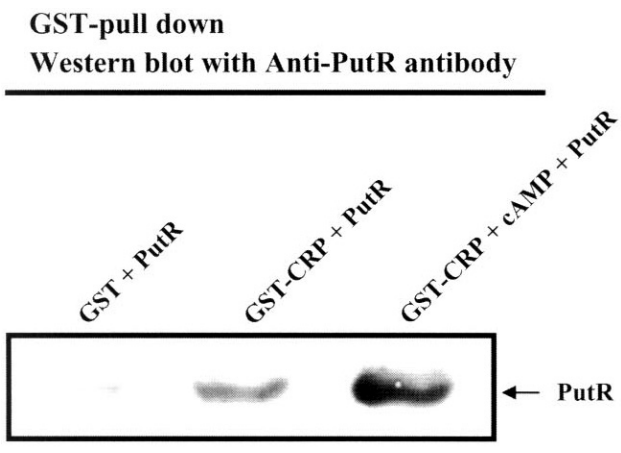

Fig. 6. PutR interacts with CRP *in vitro*.
GST-CRP or GST protein was conjugated to Glutathione-sepharose beads and incubated with 6x His-PutR protein. The binding was conducted without and with cAMP (1 mM). After extensive washing, beads were boiled in SDS gel-loading buffer and proteins were resolved in 12% SDS-PAGE. The proteins on the gel were transferred to nitrocellulose membrane for Western blot analysis.

antibody. Consistent with this, no detectable level of the *putAP* promoter fragment was detected in the anti-PutR immunoprecipitate of the *putR* mutant (Fig. 5), verifying that the PutR protein directly binds to the *putAP* promoter in *V. vulnificus* as well as *in vitro*.

PutR Interacts with CRP

In a previous report, it has been proposed that the pre-binding (or simultaneous binding) of PutR facilitates CRP to bind to the weak consensus sequences [14]. It is not yet clear how PutR facilitates the binding of CRP to the P_{put}. One possible way is that PutR recruits CRP to the binding sites by direct protein-protein interaction. To investigate if there is interaction between PutR and CRP, the specific association between CRP and PutR *in vitro* was examined using a GST-fusion protein pull-down assay. As seen in Fig. 6, the purified PutR could bind to the GST-CRP fusion protein but not to GST alone *in vitro*. When cAMP was present in the reaction, PutR bound to CRP more effectively. This result of the protein-protein interaction assay suggested that PutR can interact with CRP. An earlier report revealed that the binding sites for PutR were found to overlap with those for CRP, and the two activators were able to bind at the same time [14]. In addition to the detection of an interaction between proteins CRP and PutR *in vitro*, the close CRP and PutR binding sites suggest a possible interaction between the two proteins on the P_{put} promoter *in vivo*.

DISCUSSION

In enteric bacteria, proline is catabolized via the products of the divergently transcribed *putA* and *putP* genes [15]. The *putP* gene encodes the major proline permease, and the *putA* gene encodes a bifunctional dehydrogenase that catalyzes the oxidation of proline to glutamate [15, 18]. In enteric bacteria, the PutA protein is also involved in the transcriptional control of the *put* genes [1, 3, 21]. In the absence of proline, PutA binds to the *putA-putP* intergenic region, thereby preventing the expression of both genes. When a sufficient level of proline is available, PutA binds proline and is proposed to associate with components of the electron-transport chain in the cytoplasmic membrane, where it is enzymatically active. This releases PutA-mediated repression of *putAP* genes, allowing increased expression of both genes [16, 17]. To determine whether *V. vulnificus putAP* genes are autoregulated by PutA, we assessed the expression of *putA* by constructing a *put-lux* fusion reporter. When introduced with the reporter, the levels of luminescence in the wild-type and *putA* mutant cells were comparable. This result suggests that in contrast to the enteric bacteria, the *V. vulnificus putAP* operon is not autoregulated by the PutA protein (Fig. 2).

The analysis of the sequence downstream of the *V. vulnificus putAP* indicates the existence of a gene encoding a PutR homolog, which is a member of the Lrp family of regulatory proteins. A conserved helix-turn-helix motif located in the N-terminal part of Lrp-like regulatory proteins was also identified in the *V. vulnificus* PutR (Fig. 3). EMSA and DNase I footprinting *in vitro* revealed that PutR activates the expression of the *V. vulnificus putAP* operon by binding directly to the P_{put} promoter region [14]. However, the precise mechanism by which PutR activates expression of *putAP* was as yet uncharacterized in *V. vulnificus*. In the present study, chromatin immunoprecipitation experiments revealed that PutR binds to the P_{put} promoter DNA *in vivo*. Furthermore, specific interaction between PutR and CRP was demonstrated using a GST pull-down assay. Taken together, the possible interaction between CRP and PutR at the binding sites of the P_{put} promoter could be an explanation for the full expression of P_{put}, which is obtained only when the two proteins present together [14].

In this study, the *putAP* operon of this strain is induced by proline and activated by PutR (Figs. 2 and 4). The expression of the *A. tumefaciens* and *R. capsulates putA* genes is also regulated by PutR [4, 11]. In *R. capsulatus*, the expression of proline dehydrogenase is regulated only by the presence of proline via the regulatory gene *putR* located immediately upstream of *putA*. In the absence of proline, PutR is not able to activate the expression of *putA*. The presence of proline may cause a conformational change in the PutR protein, increasing the affinity for the *putA* promoter and, subsequently, *putA* gene expression [11]. In a similar way, the *A. tumefaciens putA* promoter is also positively regulated by the product of the regulatory gene *putR* in response to proline [4]. However, EMSA revealed that the binding affinity of PutR to the upstream region

of the P_{put} is not affected by the presence of proline, indicating that the induction effect of proline on the P_{put} activity is not mediated by PutR in *V. vulnificus* (data not shown). Undoubtedly, additional work is needed to clarify how proline contributes to the induction of P_{put}, and whether another role of proline, such as enhancing interaction between PutR and CRP, is possibly involved in the activation of P_{put}.

Acknowledgments

This study was supported by grants to S.H.C from the Korea Research Foundation (KRF-2004-005-F00054), the KOSEF (R01-2004-000-10046-0), and the Seoul Metropolitan R & D Project (10636), ROK.

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