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Coactivation of *Vibrio vulnificus putAP* operon by cAMP receptor protein and PutR through cooperative binding to overlapping sites

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Summary

The cAMP receptor protein (CRP) positively regulates the expression of *Vibrio vulnificus putAP* genes encoding a proline dehydrogenase and a proline permease. In the present study, an open reading frame encoding PutR was identified downstream of the *putAP* genes and a mutational analysis revealed that the PutR protein was also involved in regulating the *putAP* transcription by activating P_{put} promoter. Although CRP acts as a primary activator and the influence of PutR on P_{put} is mediated by CRP, the level of P_{put} activity observed when PutR and CRP functioned together was greater than the sum of P_{put} activities achieved by each activator alone. Western blot analyses demonstrated that the cellular levels of PutR and CRP were not significantly affected by each other, indicating that PutR and CRP coactivate P_{put} rather than function sequentially in a regulatory cascade. Two adjacent binding sites for PutR mapped by *in vitro* DNase I protection assays were found to overlap the CRP binding sites and were centred –91.5 (PCBI) and –133.5 bp (PCBII) upstream of the transcription start site of P_{put} respectively. PutR and CRP bind to the sites cooperatively and a dissection of the role of the binding sites revealed that CRP at PCBI plays the most crucial role in the activation of P_{put}. Accordingly, the present results revealed that PutR and CRP coactivate the expression of P_{put} and exert their effect by cooperatively binding to the promoter.

Introduction

Bacteria respond to increases in external osmolarity by actively modulating the pool of osmotically active solutes in their cytoplasm, thereby preventing the loss of water and resulting in enhanced osmotolerance (Imhoff, 1986; Csonka, 1989; Galinski, 1995; Bremer and Kramer, 2000). Members of Enterobacteriaceae accumulate large amounts organic osmolytes, known as compatible solutes or osmoprotectants, in their cytoplasm. Although a variety of novel organic osmolytes as osmoprotectants have been identified, glutamate, proline, glycine betaine, ectoine and trehalose are probably the most widely used compatible solutes in the bacteria (Csonka and Epstein, 1996; Record *et al*., 1998; Ventosa *et al*., 1998).

Vibrio vulnificus, an opportunistic Gram-negative pathogen that commonly contaminates raw oysters, is the causative agent of food-borne diseases such as gastroenteritis and life-threatening septicaemia in predisposed individuals. Mortality from septicaemia is very high (> 50%) and can occur within days from sepsis (Linkous and Oliver, 1999; Strom and Paranjpye, 2000; Gulig *et al*., 2005). Like many other pathogenic bacteria, *V. vulnificus* has to cope with ever-changing osmolarities in its growth environments. However, until now, only a few definitive analyses of the responsive adaptation of the pathogen to changes in osmolarity have been made (Kim *et al*., 2002; Lee *et al*., 2003), and thus the molecular mechanisms by which the bacterium can survive in hyperosmotic environments have not yet been well characterized.

Recently, we cloned an 8.3 kb DNA fragment of *V. vulnificus* that contains *putAP* genes encoding a proline dehydrogenase and a proline permease respectively (Kim *et al*., 2002). It was apparent that the PutP permease is the primary proline transport protein, and proline is converted into glutamate by the action of the proline dehydrogenase activity in *V. vulnificus* (Kim *et al*., 2002; Lee *et al*., 2003). A mutational analysis revealed that the gene products of *putAP* also contribute to the osmotic tolerance of *V. vulnificus* (Kim *et al*., 2002). Consistent with this, the expression of the proline dehydrogenase and accumulation of glutamate in the cells increased in response to hyperosmotic stresses, suggesting that glutamate is a compatible solute in *V. vulnificus* (Lee *et al*., 2003).

In a previous report, the transcription of the *putAP* genes was determined to be under the positive control of a cyclic AMP receptor protein (CRP) (Lee *et al*., 2003). However, no molecular analysis of the role of the CRP in the expression of *putAP* has been reported, as such the question of whether CRP directly or indirectly affects

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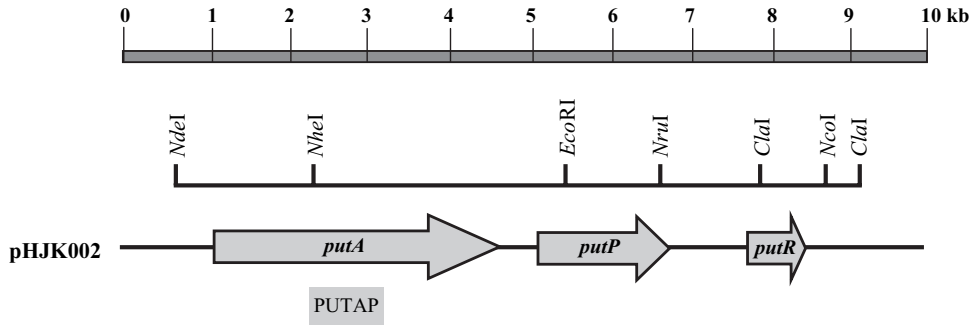

Fig. 1. Schematic representation of *V. vulnificus put* genes cloned in pHJK002. The arrows represent the transcriptional directions and coding regions of the *put* genes. The DNA probe, PUTAP, used for Northern blot analyses is depicted by a closed bar.

putAP expression has not yet been addressed. Accordingly, here we extended our efforts to elucidate the regulation of the *putAP* expression at a molecular level. First, an open reading frame (ORF), *putR*, was identified downstream of the *putAP*, and the role of the PutR protein in the regulation of the *putAP* expression was explored. The relationship between PutR and CRP was also examined by determining the proline dehydrogenase and *putA* transcript levels in *putR*, *crp* and *crp putR* backgrounds. Finally, the binding of PutR to the two adjacent binding sites upstream of the *putAP*, overlapping with the CRP binding sites, was demonstrated and the function of the binding sites and the activators for the *putAP* expression was dissected.

Results

Identification of putR *gene and construction of* putR *mutants of* V. vulnificus

In the course of a sequencing analysis of pHJK002 (Fig. 1), an ORF consisting of 164 amino acids was found downstream of the *putAP*. The deduced amino acid sequence of the ORF was 40% identical to the PutR proteins of *Agrobacterium tumefaciens* and *Rhodobacter capsulatus* (Keuntje *et al*., 1995; Cho and Winans, 1996), and thereby the ORF was named the *putR* of *V. vulnificus*. To examine the role of the PutR, the *V. vulnificus putR* mutants, in which each wild-type *putR* gene was replaced with a *putR*::*nptI* allele by homologous recombination, were constructed (data not shown). The mutants chosen for further analysis were named HJK003 for the *putR* mutant and JH031 for the *crp putR* double mutant (Table 1) respectively.

Effects of putR *or* crp *mutation on production of proline dehydrogenase*

The *putR* mutant produced approximately 1 unit of proline dehydrogenase, almost twofold lower than that produced by the wild type (Fig. 2A). The *putA* transcript was also decreased in the *putR* mutant (Fig. 2B), indicating that the proline dehydrogenase expression is positively regulated by PutR at the transcription level. The proline dehydrogenase activity and *putA* transcript in the *crp* mutant were much lower than those in the wild type (Fig. 2A and B), supporting the previous observation that CRP activates the proline dehydrogenase expression in *V. vulnificus* (Lee *et al*., 2003).

For the complementation of *putR*$^-$, plasmid pHJK0069 was constructed by subcloning the *putR* coding region, which was amplified by a polymerase chain reaction (PCR) using primers PUTR004 and PUTR005 (Table 2), into pRK415 and under an IPTG-inducible promoter (Keen *et al*., 1988). When the *putR* was induced by IPTG, the proline dehydrogenase activity and *putA* transcript of *putR*$^-$ (pHJK0069) were restored to levels comparable to, and even higher than, those in the wild type (Fig. 2A and B). Therefore, the decreased proline dehydrogenase activity of the *putR* mutant apparently resulted from the inactivation of functional *putR* rather than any polar effects on the genes downstream of the *putR*. Similar to the complementation observed with the *putR* mutant, the decreased proline dehydrogenase activity and *putA* transcript in the *crp* mutant were restored by introducing pKC0004 (Fig. 2A and B), that was constructed by subcloning the *crp* as previously described (Jeong *et al*., 2003).

It was noted that the proline dehydrogenase levels in the complemented strains *putR*$^-$ (pHJK0069) and *crp*$^-$ (pKC0004) were significantly greater than that in the wild type. The transfer and induction of recombinant *putR* and *crp* provided the respective mutant with increased levels of PutR and CRP respectively (Fig. 2C). For instance, a Western blot analysis revealed that the CRP level in the *crp*$^-$ (pKC0004) cells was relatively higher than that in the wild-type cells (Fig. 2C).

PutR and CRP coactivate expression of proline dehydrogenase

The proline dehydrogenase activities either in the *crp*$^-$,

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
KC74	ATCC29307 with *crp::nptI*; Km^r	Jeong *et al.* (2000)
DI0201	ATCC29307, *Δcrp*	Jeong *et al.* (2003)
HJK003	ATCC29307 with *putR::nptI*; Km^r	This study
JH031	ATCC29307, *Δcrp, putR::nptI*; Km^r	This study
E. coli		
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*; Km^r; host for π-requiring plasmids; conjugal donor	Laboratory collection
BL21 (DE3)	*F*$^-$, *ompT, hsdS* (r_B^-, m_B^-), *gal* (DE3)	Laboratory collection
Plasmids		
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	Keen *et al.* (1988)
pRSET-C	Expression vector, N-terminal $(His)_6$; Ap^r	Invitrogen
pKC0004	pRK415 with *crp*; Tc^r	Jeong *et al.* (2000)
pHK0011	pRK415 with promoterless *luxAB*; Tc^r	Jeong *et al.* (2000)
pHK0201	pRSET-A with *crp*; Ap^r	Choi *et al.* (2002)
pHJK002	pUC18 with *putAPR*; Ap^r	Kim *et al.* (2002)
pHJK0067	pCVD442 with *putR::nptI*; Ap^r, Km^r	This study
pHJK0069	pRK415 with *putR*; Tc^r	This study
pJH0309	pRSET-C with *putR*; Ap^r	This study
pJH0332	pHK0011 with 321 kb fragment of P_{put} upstream region; Tc^r	This study
pJH0334	pHK0011 with 232 kb fragment of P_{put} upstream region; Tc^r	This study
pJH0337	pHK0011 with 180 kb fragment of P_{put} upstream region; Tc^r	This study
pJH0338	pHK0011 with 154 kb fragment of P_{put} upstream region; Tc^r	This study

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

which carries only PutR, or in the *putR*$^-$ carrying only CRP, were significantly lower than that in the wild type (Fig. 2A), indicating that the expression of the proline dehydrogenase to the wild-type level required both PutR and CRP simultaneously. The cellular levels of PutR and CRP were determined in the same amount of total protein isolated from the wild type and its isogenic mutants (Fig. 2C). The results demonstrated that the cellular levels of PutR and CRP were not significantly affected by each other, indicating that the influence of PutR on the proline dehydrogenase activity was not the result of increasing the CRP level in the cells. Therefore, it appeared that PutR and CRP functioned cooperatively to activate the proline dehydrogenase expression rather than sequentially in a regulatory cascade. The proline dehydrogenase activity in the wild type, in which both PutR and CRP functioned together,

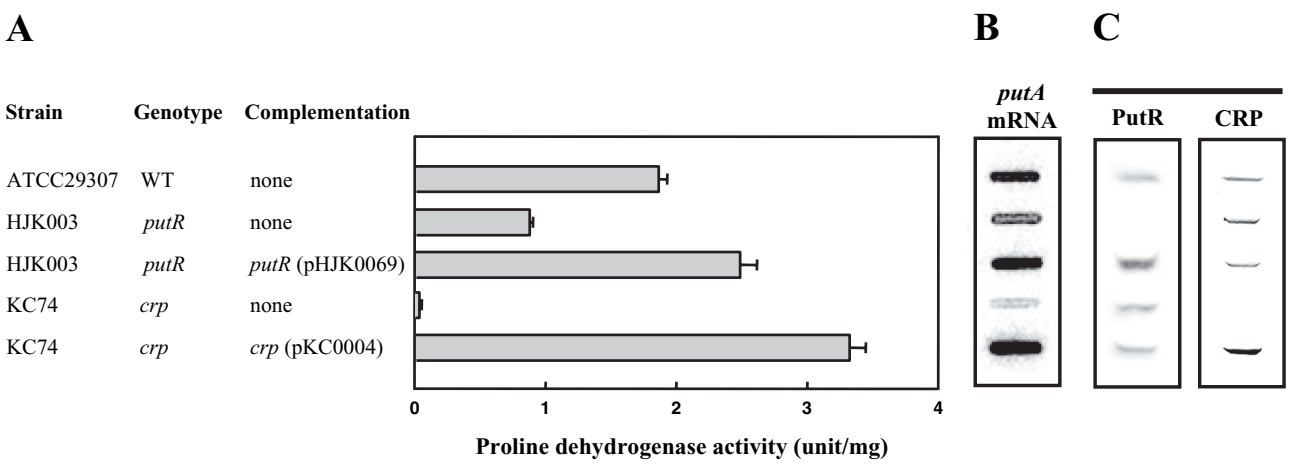

Fig. 2. Dependency of proline dehydrogenase production on PutR and CRP. Cultures of the wild type and isogenic mutants were grown in M9-P, then samples removed at an OD_{600} of 0.8 were analysed for their proline dehydrogenase activity (A), *putA* transcript (B) and PutR or CRP levels (C). Details for determining the proline dehydrogenase activity, a Northern slot blot of the *putA* transcript, and Western blot of PutR and CRP are described in *Experimental procedures*. For complementation tests, when the cultures reached an OD_{600} of 0.5, IPTG was added to a final concentration of 0.1 mM to induce the expression of recombinant *putR* (i.e. on pHJK0069) or *crp* (i.e. on pKC0004), as indicated. Error bars represent SEM.

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Oligonucleotide sequence, 5′→3′[a]	Location[b]	Use
PUTA004	CATGAATTCCTCCTTATGCGTTCTCAGTC	*putA*	Amplification of *putA*
PUTA005	ACTCTAGATTCGGGGATGAGATTGAGGAAA	*putA*	Amplification of *putA*
PUTA0012	GAGATCAATGCCCATAGCTTATCGAGC	*putA*	Primer extension
PUTA0313	CCACTCCTTTACTCGCTTACAG	–181 to –203	Binding assay
PUTA0314	AACTCCGGCTTTAACACATCT	64–84	Binding assay
PUTA0011	TTAGGATCCATCAATGCCCATAGCTTA	103–120	Promoter deletion
PUTA0315	TTAGGTACCCACTCCTTTACTCGCTT	–185 to –201	Promoter deletion
PUTA0316	ATTGGTACCTTGCCTAAATTCGTGTG	–96 to –112	Promoter deletion
PUTA0317	AATGGTACCTAGCAAAAAGCTCGGAG	–44 to –60	Promoter deletion
PUTA0318	TAAGGTACCAGTAATCCTCTCAGGCTC	–17 to –34	Promoter deletion
PUTR004	CTGAATTCTCCCTCTTCTATTTTGGCG	*putR*	Amplification of *putR*
PUTR005	TAATCTAGAAAATGATAAGGGACGAAGGATG	*putR*	Amplification of *putR*
HIS-PUTR007	TAACTGCAGAATGATAAGGGACGAAGGATG	*putR*	Amplification of *putR*
HIS-PUTR008	ATTAAGCTTGCTGCGTTACTTTTGGTACAA	*putR*	Amplification of *putR*

a. Regions of oligonucleotides not complementary to corresponding genes are underlined.
b. Where the nucleotides were hybridized to.

was even greater than the sum of the proline dehydrogenase activity achieved by each activator alone (Fig. 2A). Therefore, these combined results suggest that PutR and CRP coactivate the proline dehydrogenase expression and function synergistically.

Effects of putR *or* crp *mutation on activity of* putAP *promoter*

The activities of the *putAP* promoter, P_{put}, in the wild type and isogenic mutants were compared using primer extension analyses. A reverse transcript was identified from the RNA isolated from the wild-type cells (Fig. 3). The 5′-end of the *putAP* transcript, located 48 bp upstream of the translational initiation codon of the *putA* gene, was subsequently designed +1 (Figs 3 and 9).

When compared with the wild type, a decreased reverse transcript was apparent with the RNA from the *putR* mutant (Fig. 3). Primer extension analyses performed with the RNA prepared from cells of the *crp* mutant, produced almost undetectable product (Fig. 3). These results suggest that the PutR- or CRP-dependent variation of the proline dehydrogenase activity and the *putA* transcript level (Fig. 2A and B) resulted from changes in the P_{put} activity. Determined based on the intensity of the bands of the reverse transcripts, the decreased P_{put} activities in the *putR* and *crp* mutants were restored by the introduction of the recombinant *putR* and *crp* respectively (Fig. 3). The general patterns and magnitudes of the restoration of the P_{put} activities were similar to those of the proline dehydrogenase activities, which were determined directly (Figs 2A and 3).

Effect of PutR on P_{put} *activity is mediated through CRP*

The proline dehydrogenase activity and *putA* transcript level in the *crp putR* double mutant were indistinguishable from those in the *crp* single mutant (Figs 2 and 4), indi-

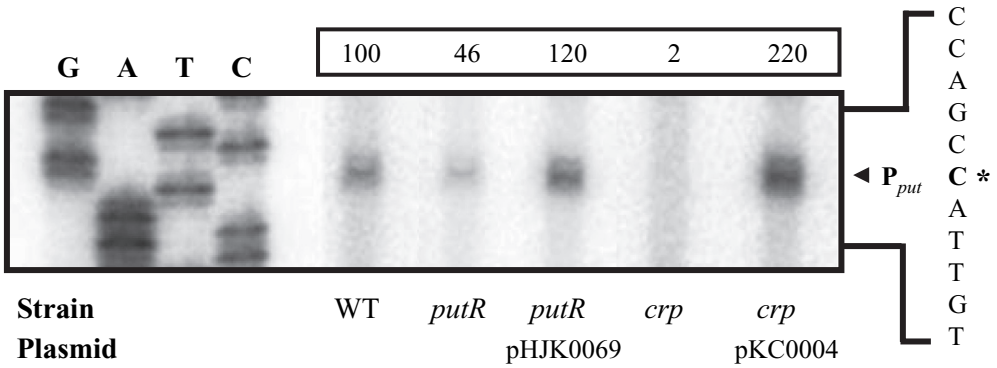

Fig. 3. Activities of P_{put} promoter in *V. vulnificus* with different genetic backgrounds. The P_{put} activities were determined separately by primer extension of the RNA derived from the wild type, isogenic mutants and complemented strains, as indicated. Total RNA was prepared from each culture at an OD_{600} of 0.8. Lanes G, A, T and C represent the nucleotide sequencing ladders of pHJK002. The asterisk indicates the site of the transcription start for P_{put}. The relative levels of the P_{put} activity are presented relative to the level of the P_{put} activity in wild type. WT, wild type; *putR*, *putR* mutant; *crp*, *crp* mutant.

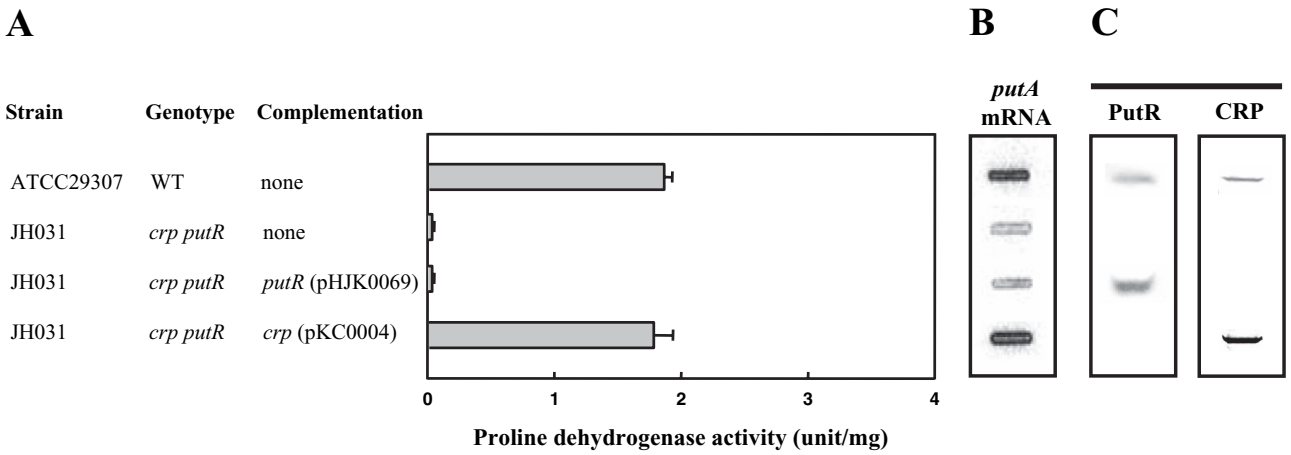

Fig. 4. PutR effect on P_{put} activity is mediated by CRP. Samples were removed from cultures of the wild type and isogenic mutants grown to an OD_{600} of 0.8, and analysed to determine the proline dehydrogenase activity (A), *putA* transcript (B) and PutR or CRP levels (C). The complementation tests and the determination of the proline dehydrogenase activity, the *putA* transcript, PutR and CRP levels were performed by the same procedures as in Fig. 2. Error bars represent SEM.

cating that the additional inactivation of the *putR* had no influence on the P_{put} activity in the absence of CRP. The transfer and induction of recombinant *putR* provided the *crp putR* double mutant with an increased PutR. A Western blot analysis revealed that the PutR level in the *crp*$^-$ *putR*$^-$ (pHJK0069) cells was relatively higher than that in the *crp*$^-$ cells (Figs 2C and 4C). Again, the proline dehydrogenase activity and the *putA* transcript level in the *crp*$^-$ *putR*$^-$ (pHJK0069) were almost identical to those in the *crp* mutant (Figs 2 and 4). From these results, it was apparent that a variation in the PutR cellular level did not affect the P_{put} activity unless CRP was also present. The results indicated that the effect of PutR was mediated through CRP, thereby suggesting that CRP is the primary regulator for the activation of P_{put}.

PutR and CRP are required simultaneously for full activity of P_{put}

To determine if an increased amount of CRP would compensate for a lack of PutR in the activation of P_{put}, the *crp* expression plasmid pKC0004 was introduced into the *crp putR* double mutant. When *crp* was induced by IPTG, the CRP cellular level in *crp*$^-$*putR*$^-$ (pKC0004) was higher than that in the *putR* single mutant (Figs 2C and 4C). The proline dehydrogenase and the *putA* transcript level in *crp*$^-$*putR*$^-$ (pKC0004) were comparable to those in the wild type (Fig. 4A and B), indicating that CRP, when overproduced, was able to activate P_{put} to the wild-type level in the absence of PutR. However, the proline dehydrogenase level in *crp*$^-$*putR*$^-$ (pKC0004) is still lower than that in *crp*$^-$ (pKC0004) (Figs 2A and 4A). Because the CRP levels were similar in the *crp*$^-$*putR*$^-$ (pKC0004) and *crp*$^-$ (pKC0004), but PutR is absent in the *crp*$^-$*putR*$^-$ (pKC0004), it was apparent that the lower proline dehydrogenase level observed in *crp*$^-$*putR*$^-$ (pKC0004) was solely due to the lack of PutR. Thus, even though the overproduced CRP was seemingly able to compensate for the lack of PutR, PutR and CRP were required simultaneously for the full activation of P_{put}.

PutR and CRP bind cooperatively to P_{put}

In gel-mobility shift assays, the addition of PutR at a concentration of 50 nM resulted in a shift of the 287 bp DNA fragment carrying the P_{put} regulatory region (from –203 to +84) to a single band with a slower mobility (Fig. 5A). The binding of PutR was also specific, because assays were performed in the presence of 1 μg of poly (dI-dC) as a non-specific competitor. The result of the gel-mobility shift assay with PutR indicated that PutR binds to either a single binding site or to multiple binding sites with a comparable affinity. Based on the concentration of PutR that was required to retard 50% of the labelled probe, it was estimated that the dissociation binding constant (K_d) for PutR was approximately 190 nM (Fig. 5C).

When PutR and CRP were present simultaneously in the reaction, most of each band representing the DNA bound with either PutR (lane 2) or CRP (lane 3) disappeared, whereas a slower-moving band representing the DNA bound with both proteins was observed (lanes 4, 5 and 6, Fig. 5B). The K_d for PutR was approximately 60 nM in the presence of 100 nM CRP (Fig. 5C). This K_d for PutR was lower than the K_d, which was approximately 190 nM as determined in the absence of CRP. These results suggested that there is a cooperative binding between PutR and CRP to the P_{put} promoter.

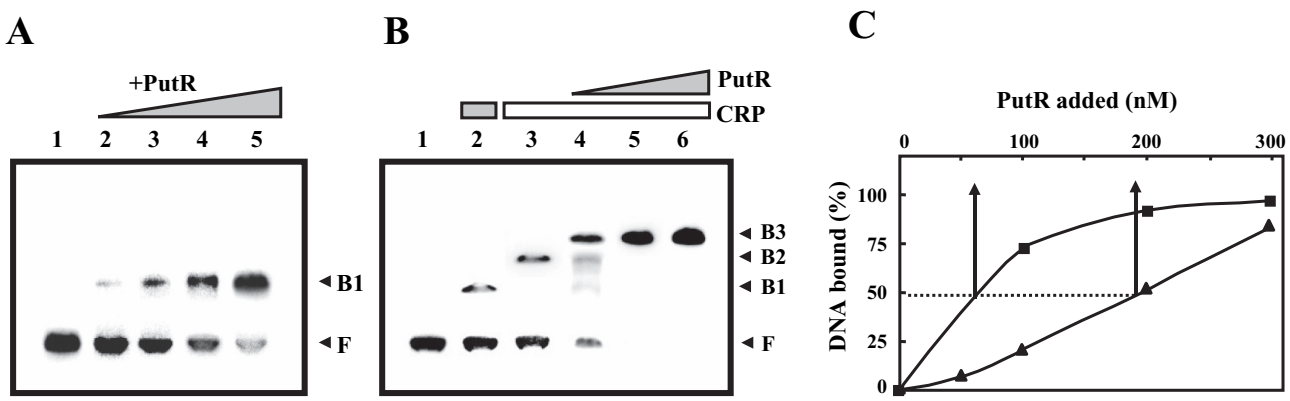

Fig. 5. Gel-mobility shift assay for binding of PutR to P_{put} regulatory region.
A and B. A 287 bp DNA fragment of the upstream region of P_{put} was radioactively labelled and then used as a probe DNA. The radiolabelled fragments were mixed with increasing amounts of PutR in the absence of CRP (A) or in the presence of 100 nM CRP (B). For A, 0, 50, 100, 200, 300 nM of PutR in lanes 1–5 respectively. For B, lane 1, no protein; lane 2, 100 nM PutR; lanes 3–6 (in the presence of 100 nM CRP), 0, 100, 200, 300 nM PutR respectively. The reaction mixtures were resolved on a 5% polyacrylamide gel. For the binding of CRP, cAMP was included in all the reaction mixtures at a final concentration of 1 mM. The positions of the unbound fragments (F), the fragments retarded by PutR (B1), CRP (B2) or mixture of PutR and CRP (B3), are indicated by arrows.
C. The relative affinities of PutR for the upstream region of P_{put} in the absence (▲) or presence (■) of CRP were compared using the data from A and B respectively. The concentration of bound DNA was calculated and plotted against the concentration of the PutR added. Each arrow points to the position of half-maximal binding corresponding to the K_d.

In similar DNA-binding assays, CRP also displayed specific binding to the P_{put} regulatory region. As seen in Fig. 6A, the P_{put} regulatory region formed an intermediate band that was chased away to a slower migrating band at higher concentrations of the protein. This pattern of migration suggested that at least two binding sites with different affinities for CRP are present in the P_{put} regulatory region.

In a second gel-mobility shift assay, CRP was added to the DNA which was preincubated with 100 nM PutR (Fig. 6B). The PutR was also found to facilitate CRP to bind to the DNA. The K_d for CRP, when incubated in the presence of PutR, was approximately 30 nM, which was fourfold lower than the K_d as determined by incubating CRP alone with the P_{put} DNA.

From these results, it is apparent that PutR and CRP activate P_{put} by directly binding to the promoter. These results also suggested that the PutR and CRP bind to the P_{put} DNA more effectively when they are present together, and that there is some cooperative interaction involved in their binding to the DNA.

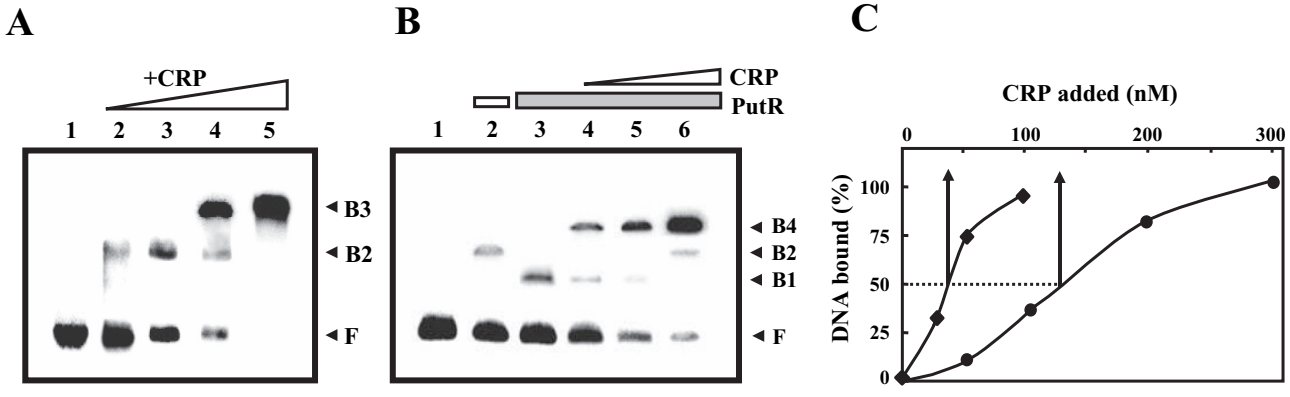

Fig. 6. Gel-mobility shift assay for binding of CRP to P_{put} regulatory region.
A and B. Gel-mobility shift assays were performed under the same conditions as in Fig. 5, except that increasing amounts of CRP (A), or mixtures of 100 nM PutR and increasing amounts of CRP, were added to the ^{32}P-labelled 287 bp P_{put} promoter as indicated. For A, 0, 50, 100, 200, 300 nM of CRP in lanes 1–5 respectively. For B, lane 1, no protein; lane 2, 50 nM CRP; lanes 3–6 (in the presence of 100 nM PutR), 0, 30, 50, 100 nM CRP respectively. The positions of the unbound fragments (F), the fragments retarded by PutR (B1), CRP (B2 and B3) or mixture of PutR and CRP (B4), are indicated by arrows.
C. The relative affinities of CRP for the upstream region of P_{put} in the absence (●) or presence (◆) of PutR were compared using the data from A and B, respectively, and presented as described in Fig. 5.

Identification of binding sites for PutR and CRP using DNase I protection analysis

As shown in Fig. 7A, the DNase I footprinting performed with PutR revealed two clear protection patterns in the upstream region of P_{put} extending from –97 to –80 and from –139 to –123 respectively (Fig. 7A and 9). Both sequences were equally protected by the same level of PutR, indicating that PutR bound to the two sites with a comparable affinity. The pattern of protection was consistent with the result of gel-mobility shift assays where only a single DNA–PutR complex was produced (Fig. 5A).

When the sequences for the binding of CRP to the P_{put} promoter were mapped with CRP up to 300 nM, the CRP footprint extended from –103 to –79 (Figs 7B and 9). When increasing the CRP, another region extending from –143 to –122 was protected from DNase I digestion (Figs 7C and 9). This sequential protection of P_{put} with increasing CRP was consistent with the previous observation that at least two binding sites with different affinities for CRP are present in the P_{put} regulatory region (Fig. 6A). The binding sequences overlapped with the sequences for the binding of PutR. The regions extending from –103 to –79 (centred at –91.5) and from –143 to –122 (centred at –133.5) were named PCBI and PCBII, to represent the PutR/CRP binding site I and II respectively.

To further examine the cooperative interaction between PutR and CRP, a DNase I protection assay was performed by adding PutR and CRP to the P_{put} promoter DNA at the same time. When 150 nM of both PutR and CRP were added simultaneously to the DNA, the protection was more complete than the protection obtained by adding 300 nM of either PutR or CRP alone (Fig. 7D). The results supported our previous hypothesis that there is a cooperative binding of PutR and CRP to the P_{put} promoter.

Dissection of the role of activators and binding sites in activation of P_{put}

To dissect the role of each activator on either PCBI or PCBII in the activation of the P_{put} promoter, the *put–luxAB* transcriptional fusions were transferred into the wild type

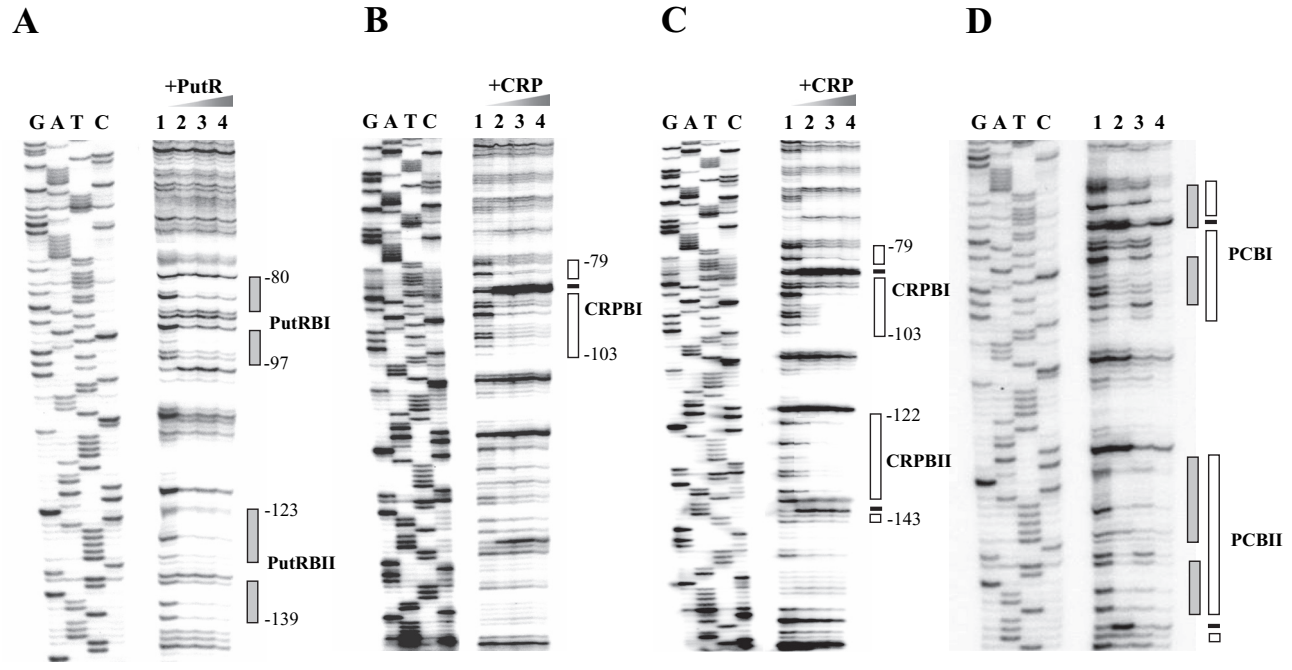

Fig. 7. Identification of binding sites for PutR and CRP.
A. DNase I protection analysis of PutR binding to P_{put} regulatory region. The ^{32}P-labelled 287 bp P_{put} promoter was incubated with increasing amounts of PutR, then digested with DNase I. Lane 1, no PutR added; lanes 2–4, PutR at 300 nM, 400 nM and 500 nM respectively. The protection by PutR is indicated by shaded boxes (PutRBI and II).
B and C. To analyse the CRP binding to the P_{put} promoter using DNase I footprinting, the same experimental conditions were used, except that 1 mM cAMP was added. For B, lane 1, no CRP added; lanes 2–4, CRP at 100 nM, 200 nM and 300 nM respectively. For C, Lane 1, no CRP added; lanes 2–4, CRP at 300 nM, 400 nM and 500 nM respectively. The nucleotides showing an enhanced cleavage in the presence of CRP are indicated by thick lines, while the regions protected by CRP are indicated by open boxes (CRPBI and II).
D. The DNase I protection assay was performed by the same procedure as in (B), except that no protein (lane 1), 300 nM CRP (lane 2), 300 nM PutR (lane 3), or a mixture of 150 nM PutR and 150 nM CRP (lane 4), was added to the ^{32}P-labelled 287 bp P_{put} promoter as indicated. For all panels, lanes G, A, T and C represent the nucleotide sequencing ladders of pHJK002. PCBI, PutR/CRP binding site I; PCBII, PutR/CRP binding site II.

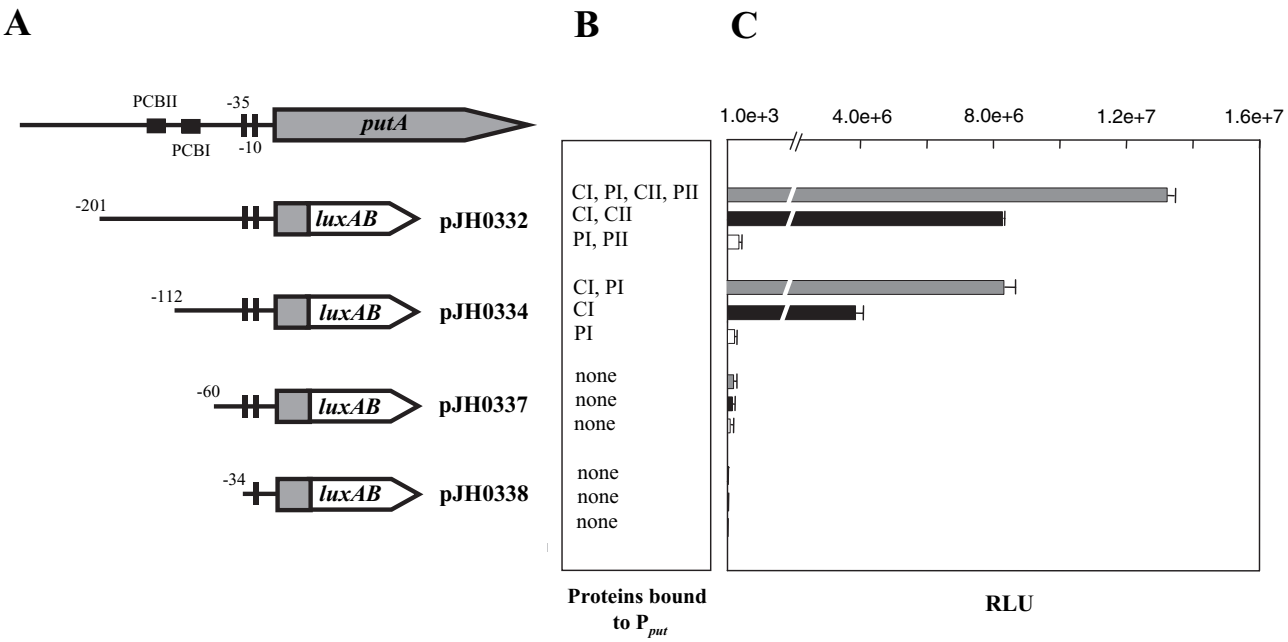

Fig. 8. Dissection of the role of activators and binding sites in activation of P_{put}.
A. Construction of *put–lux* fusion pJH-plasmids. PCR fragments carrying the P_{put} regulatory region with 5′-end deletions were subcloned into pHK0011 (Choi *et al*., 2002) to create each pJH-reporter. Solid lines, the upstream region of P_{put}; shaded blocks, the *putA* coding region; open blocks, the *luxAB*. The wild-type P_{put} regulatory region is shown on top with the proposed –10 and –35 regions, and the binding sites PCBI and PCBII respectively.
B. Proteins presumably bound to the PCBI and PCBII binding sites carried in the P_{put} regulatory region in each pJH-reporter. CI, CRP in PCBI; CII, CRP in PCBII; PI, PutR in PCBI; PII, PutR in PCBII.
C. Cellular luminescence determined from wild type (shaded bars), *putR* isogenic mutant (filled bars), and *crp* isogenic mutant (open bars) containing each pJH-reporter as indicated. Cultures in the exponential phase of growth (OD_{600} of 0.8) were used to measure the cellular luminescences. Error bars represent the SEM. RLU, arbitrary relative light units.

and isogenic mutants (Fig. 8A). For the wild-type strain containing pJH0332, a plasmid carrying the regulatory region with both PCBI and PCBII, the luminescence activity was about 1.3×10^7 relative light units (RLU) (Fig. 8C). The light produced by *putR*$^-$ with pJH0332 was reduced to 8×10^6 RLU. Compared with this, *crp*$^-$ containing pJH0332 produced approximately 1000-fold less luminescence (Fig. 8C). In the wild-type cells, both PutR and CRP probably could bind to PCBI and PCBII, yet, in contrast, only CRP or only PutR could occupy the binding sites in *putR*$^-$ or *crp*$^-$ respectively (Fig. 8B). Again, these results supported our previous assumption that CRP is the primary regulator and that PutR plays a secondary role in the activation of P_{put}.

Luminescence was also determined in the strains that carried pJH0334 or pJH0337 (Fig. 8A and 8C). In wild-type cells with pJH0334 carrying only PCBI (Fig. 8A), CRP and PutR presumably bound to PCBI and the luminescence level was approximately half of the luminescence with pJH0332. However, when transformed with pJH0337 carrying no binding sites (Fig. 8A), the luminescence level in the wild type decreased more than 1000-fold, close to the basal level (i.e. the luminescence from pJH0338) (Fig. 8C). These results suggested that PCBII is a secondary binding site while PCBI is the crucial primary binding site for the activation of P_{put}.

When transferred into *putR*$^-$, both PCBI and PCBII in pJH0332, only PCBI in pJH0334, or no binding sites in pJH0337 could be occupied by CRP (Fig. 8A and B). The level of luminescence in *putR*$^-$ with pJH0334 was approximately half of that with pJH0332. In contrast, the level of luminescence with pJH0337 decreased close to the basal level (Fig. 8C). These results indicated that CRP in PCBI plays more important role than CRP in PCBII in the activation of P_{put}.

In summary, it appeared that PutR acts as a secondary activator and exerts its effect through CRP, the primary activator for the regulation of P_{put}. PutR and CRP coactivate the P_{put} activity rather than function sequentially in a regulatory cascade. PutR and CRP affect the P_{put} activity by cooperatively binding to two binding sites PCBI and II. Finally, CRP in PCBI plays the most important role in the activation of P_{put}.

Discussion

The genetic organization and regulatory mechanisms that control the expression of the *putAP* genes are quite dif-

ferent among the members of Gram-negative Enterobacteriaceae. In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, the *putA* and *putP* genes are transcribed divergently (Maloy, 1987; Vilchez *et al*., 2000). However, the transcription orientations of the *putAP* genes of *V. vulnificus* are in the same direction rather than divergent (Kim *et al*., 2002). In *E. coli* and *S. enterica* serovar Typhimurium, the PutA protein is a bifunctional enzyme; in addition to its enzymatic activities, PutA functions as a proline-responsive repressor of the *putA* and *putP* genes (Maloy and Roth, 1983; Allen *et al*., 1993; Maloy and Stewart, 1993; Ostrovsky and Maloy, 1993; Muro-Pastor *et al*., 1997). Because the genetic organization differs from that of the *putAP* of the enteric bacteria, it is not surprising that the *putAP* of *V. vulnificus* is probably not expressed and modulated by the same way as observed in the enteric bacteria. Consistent with this, the *V. vulnificus putA* was not autoregulated by the PutA protein (data not shown). Instead, the expression of the *V. vulnificus putA* was induced in response to hyperosmotic stresses (Lee *et al*., 2003).

The amino acid sequence and molecular weight of the *V. vulnificus* PutR are similar to those of the PutR from *R. capsulatus* and *A. tumefaciens* (Keuntje *et al*., 1995; Cho and Winans, 1996). In *R. capsulatus* and *A. tumefaciens*, where the *putA* is organized as a monocistroinc transcriptional unit and the *putP* is not located adjacent to the *putA*, the *putR* transcribed divergently from the *putA* (Keuntje *et al*., 1995; Cho and Winans, 1996). In these bacteria, disruption of the *putR* abolished the induction of the *putA* promoter by proline, indicating that PutR is absolutely required for *putA* expression (Keuntje *et al*., 1995; Cho and Winans, 1996). In contrast to this, the data in the present study demonstrated that the *V. vulnificus* PutR acts as a secondary regulator and the activation of P_{put} by PutR is mediated by CRP. Although CRP is the primary activator for the expression of P_{put}, the CRP binding sites reveal weak homologies to a consensus sequence for CRP binding (TGTGAN6-8TCACA, Botsford and Harman, 1992) (Fig. 9). The binding site PCBI only scores a 50% homology to the consensus sequence, and there are no identifiable consensus-like sequence elements for CRP binding at PCBII (Fig. 9). Therefore, it is likely that the prebinding (or simultaneous binding) of PutR facilitates CRP to bind to these weak consensus sequences. This hypothesis is supported by the observation that CRP bound to the binding sites with a higher affinity in the presence of PutR as determined by a gel-mobility shift assay and a DNase I protection assay (Figs 6B and 7D).

It is not yet clear how PutR facilitates the binding of CRP to the P_{put}. One possible way is that PutR recruits CRP at the binding sites by direct protein–protein interaction. The binding sites for PutR were found to overlap with those for CRP, and the two activators were able to bind at the same

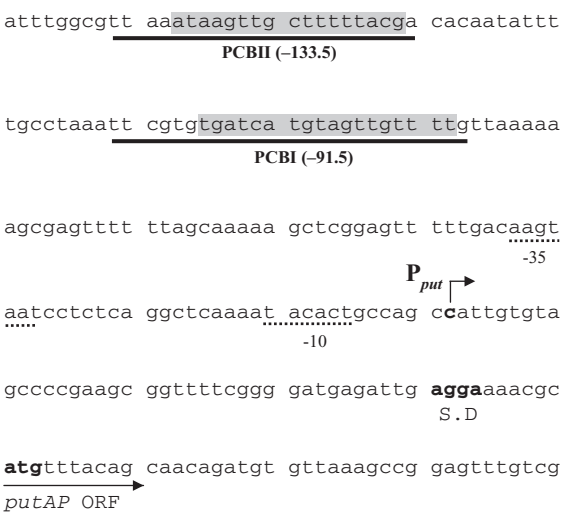

Fig. 9. Sequence analysis of P_{put} upstream region. The transcription start site is indicated by a bent arrow. The positions of the putative –10 and –35 regions are underlined with dashed lines for the promoter P_{put}. The sequences proposed for the binding sites of PutR and CRP, termed PCBI and II, are represented by shaded boxes (for PutR) and lines (for CRP). The ATG translation initiation codon and putative ribosome-binding site (AGGA) are indicated in boldface.

time (Figs 5B, 6B and 7D), indicating that the two activators might bind to opposite faces of the DNA helix. Relatively few examples of the coregulation of bacterial promoters by multiple activators present at overlapped binding sites have been reported (Buchet *et al*., 1999). Nonetheless, the CRP and PutR at the binding sites of the P_{put} promoter may be close enough to suggest a possible interaction between the two proteins. Another way is that PutR alters the conformation of the DNA helix in and around the binding sites, making it better recognized by CRP. It has already been demonstrated that transcription initiation at many bacterial promoters is modulated by the nucleoid proteins, such as Lrp and IHF, that induce conformational changes in the DNA helix (McLeod and Johnson, 2001). It is noteworthy that a conserved DNA-binding helix-turn-helix motif (Keuntje *et al*., 1995; http://www.expasy.org/prosite/) located in the N-terminus of Lrp-like regulatory protein is also identified in the *V. vulnificus* PutR (data not shown).

Another interesting feature of the P_{put} is the presence of two adjacent binding sites, PCBI and PCBII, for PutR and CRP. Although the PCBI appeared to have a crucial effect on the activation of P_{put}, PCBII also showed a distinct role, and the deletion of the PCBII decreased the expression of P_{put} (Fig. 8). It is unclear how PutR and CRP at the PCBII act in concert with the regulators at the PCBI for the activation of P_{put}. The PCBI and PCBII are separated from one another by more than 40 bp (Fig. 9). Nonetheless, this distance is not unusual for direct interaction between the proteins at the two binding sites, especially

in the presence of nucleoid proteins (Barnard *et al*., 2004). Therefore, two types of interaction are possibly working for the activation of P_{put}; one is between PutR and CRP at a binding site and the other is between the two adjacent binding sites (and the regulators at the sites) respectively. Undoubtedly, additional work is needed to clarify how PutR collaborates with CRP for the full activation of P_{put}, and whether direct interaction between the regulators bound to PCBI and PCBII is really involved in the activation of P_{put}. However, utilization of the two binding sites with differential affinities for the activators may permit precise adjustment of the *put* expression in response to environmental signals such as changes in osmolarity.

Experimental procedures

Strains, plasmids and culture media

The strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the *V. vulnificus* strains were grown in Luria–Bertani medium supplemented with 2.0% (w/v) NaCl (LBS). When required, modified M9 (Sambrook and Russell, 2001), M9-P, in which 100 mM proline was supplemented, was used.

Generation of putR::nptI *mutants*

To inactivate *putR in vitro*, a 1.2 kb *nptI* DNA conferring resistance to kanamycin (Oka *et al*., 1981) was inserted into a unique ClaI site present within the *putR* coding region to result in pHJK0067 (Table 1). To generate the *putR::nptI* mutant by homologous recombination, *E. coli* SM10 λ *pir*, *tra* (containing pHJK0067) (Miller and Mekalanos, 1988) was used as a conjugal donor to *V. vulnificus* ATCC29307. For construction of the *crp putR* double mutant, *V. vulnificus* DI0201, an isogenic *crp* deletion mutant of ATCC29307 (Jeong *et al*., 2003), was used as a recipient. The conjugation and isolation of the transconjugants were conducted using previously described methods (Jeong *et al*., 2000; Jeong *et al*., 2001).

Measurement of proline dehydrogenase activities

Cultures of *V. vulnificus* strains in M9-P broth were grown at 30°C with aeration and samples were removed at an OD_{600} of 0.8 for determination of the proline dehydrogenase activity. The proline dehydrogenase activities were determined as previously described (Kim *et al*., 2002). A unit of the enzyme activity was defined by the method of Ostrovsky (Ostrovsky *et al*., 1991). The protein concentrations were determined by the method of Bradford (1976). Averages and standard errors of the mean (SEM) were calculated from at least three independent trials.

RNA purification and analysis of putA *transcripts*

The total cellular RNA from the *V. vulnificus* strains was isolated using a Trizol reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. For the primer extension experiments, an end-labelled 27-base primer PUTA0012 (Table 2) complementary to the coding region of the *putA* was added to the RNA and then extended with SuperScript II RNase H^- reverse transcriptase (Invitrogen) as previously described (Jeong et al., 2001; 2003; Choi et al., 2002). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated from pHJK002 (Table 1) with the same primer used for the primer extension.

For the Northern slot blot analyses (Jeong *et al*., 2001), a 960 bp DNA fragment, containing the coding region of the *putA*, was amplified by a PCR using the primers PUTA004 and PUTA005 (Table 2), labelled with [α-^{32}P] dCTP, named PUTAP, and then used for the hybridizations. The primer extension products and Northern hybridization blots were visualized and quantified using a phosphorimage analyser (BAS1500, Fuji Photo Film, Tokyo, Japan) and the Image Gauge (version 3.12) program.

Overexpression and purification of V. vulnificus *PutR and CRP*

The *putR* coding region was amplified by a PCR using the primers, HIS-PUTR007 and HIS-PUTR008 (Table 2), and was then subcloned into a His_6 tagging expression vector, pRSET-C (Invitrogen), to result in pJH0309 (Table 1). The His-tagged PutR protein was then expressed in *E. coli* BL21 (DE3), and purified by affinity chromatography according to the manufacturer's procedure (QIAGEN, Valencia, CA). In a similar way, the expression and purification of the His-tagged CRP were carried out using pHK0201, carrying the *V. vulnificus crp* gene, as described elsewhere (Choi *et al*., 2002).

Western blot analysis of V. vulnificus *PutR and CRP*

The purified His-tagged proteins were used to raise polyclonal antibodies to the PutR and CRP of *V. vulnificus* as previously described (Jeong *et al*., 2003). For the Western immunoblotting, the cellular proteins of the wild type and its isogenic mutants were resolved by SDS-PAGE (Laemmli, 1970). The resolved proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and probed with a 1:5000 dilution of the rat polyclonal antibodies. The bound antibodies were detected using goat anti-rat IgG conjugated with alkaline phosphatase (Sigma, St Louis, MO), and visualized by incubation with a 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate (Sigma) (Jeong *et al*., 2000).

Gel-mobility shift assay and DNase I footprinting

The 287 bp upstream region of the *putAP*, extending from residues −203 to +84, was amplified by a PCR using ^{32}P-labelled PUTA0313 and unlabelled PUTA0314 as the primers

(Table 2). The binding of CRP to the labelled DNA and electrophoretic analysis of the DNA–CRP complexes have already been described (Choi *et al*., 2002). The protein-DNA binding reactions with PutR were the same as those with CRP, except that cAMP was omitted from the reaction buffer.

The same labelled 287 bp DNA was used for the DNase I protection assays. The binding of CRP to the labelled DNA, and DNase I digestion of the DNA–CRP complexes followed the procedures previously described by Choi *et al*. (Choi *et al*., 2002). After precipitation with ethanol, the digested-DNA products were resolved on a sequencing gel alongside sequencing ladders of pHJK002 generated using PUTA0313 as the primer. Similar experimental conditions were used with PutR, except that cAMP was omitted from the reaction buffer, for the DNA–PutR complex formation. The gels were visualized as described above for the Northern analysis.

Construction of set of put–luxAB transcriptional fusions

The primer PUTA0011 (Table 2) was used in conjunction with one of the following primers to amplify the DNA upstream of the *putAP*: PUTA0315 (for pJH0332), PUTA0316 (for pJH0334), PUTA0317 (for pJH0337) or PUTA0318 (for pJH0338) (Table 2). The primers were designed to amplify the P_{put} promoter region extending up to −201, −112, −60 and −34 respectively (Fig. 8A). The DNA fragments were inserted into pHK0011, which carries promoterless *luxAB* luciferase genes (Jeong *et al*., 2001), to create four *put–luxAB* reporter constructs, as confirmed by DNA sequencing. The *put–luxAB* reporters were then transferred into wild type and the isogenic mutants by conjugation. The cellular luminescence of the cultures was measured with a luminometer (Lumat Model 9507, Berthold, Germany) and expressed in arbitrary RLU as previously described (Choi *et al*., 2002; Jeong *et al*., 2003).

Nucleotide sequence accession number

The nucleotide sequence of *putR* gene of *V. vulnificus* ATCC29307 was deposited into the GenBank under Accession number AF 454004.

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References

Allen, S.W., Senti-Willis, A., and Maloy, S.R. (1993) DNA sequence of the *putA* gene from *Salmonella typhimurium*: a bifunctional membrane-associated dehydrogenase that binds DNA. *Nucleic Acids Res* **21:** 1676.

Barnard, A., Wolfe, A., and Busby, S. (2004) Regulation at complex bacterial promoters: how bacteria use different promoter organizations to produce different regulatory outcomes. *Curr Opin Microbiol* **7:** 102–108.

Botsford, J., and Harman, J.G. (1992) Cyclic AMP in prokaryotes. *Microbiol Rev* **56:** 100–122.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* **72:** 248–254.

Bremer, E., and Kramer, R. (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. In *Bacterial Stress Responses.* Storz, G., and Hengge-Aronis, R. (eds). Washington, DC: American Society for Microbiology Press, pp. 79–98.

Buchet, A., Nasser, W., Eichler, K., and Mandrand-Berthelot, M. (1999) Positive co-regulation of the *Escherichia coli* carnitine pathway *cai* and *fix* operons by CRP and the CaiF activator. *Mol Microbiol* **34:** 562–575.

Cho, K., and Winans, S.C. (1996) The *putA* gene of *Agrobacterium tumefaciens* is transcriptionally activated in response to proline by an Lrp-like protein and is not autoregulated. *Mol Microbiol* **22:** 1025–1033.

Choi, H.K., Park, N.Y., Kim, D., Chung, H.J., Ryu, S., and Choi, S.H. (2002) Promoter analysis and regulatory characteristics of *vvhBA* encoding cytolytic hemolysin of *Vibrio vulnificus*. *J Biol Chem* **277:** 47292–47299.

Csonka, L.N. (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Rev* **53:** 121–147.

Csonka, L.N., and Epstein, W. (1996) Osmoregulation. In Escherichia coli *and* Salmonella typhimurium*: Cellular and Molecular Biology*, 2nd edn. Neidhardt, F.C., Curtis, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., *et al.* (eds). Washington, DC: American Society for Microbiology Press, pp. 1210–1223.

Galinski, E.A. (1995) Osmoadaptation in bacteria. *Adv Microb Physiol* **37:** 273–328.

Gulig, P.A., Bourdage, K.L., and Starks, A.M. (2005) Molecular pathogenesis of *Vibrio vulnificus*. *J Microbiol* **43:** 118–131.

Imhoff, J.F. (1986) Osmoregulation and compatible solutes in eubacteria. *FEMS Microbiol Rev* **39:** 57–66.

Jeong, H.S., Jeong, K.C., Choi, H.K., Park, K.J., Lee, K.H., Rhee, J.H., and Choi, S.H. (2001) Differential expression of *Vibrio vulnificus* elastase gene in a growth phase-dependent manner by two different types of promoters. *J Biol Chem* **276:** 13875–13880.

Jeong, H.S., Lee, M.H., Lee, K.H., Park, S.J., and Choi, S.H. (2003) SmcR and cyclic AMP receptor protein coactivate *Vibrio vulnificus vvpE* encoding elastase through the RpoS-dependent promoter in a synergistic manner. *J Biol Chem* **278:** 45072–45081.

Jeong, K.C., Jeong, H.S., Lee, S.E., Chung, S.S., Rhee, J.H., Starks, A.M., *et al.* (2000) Construction and phenotypic evaluation of a *Vibrio vulnificus vvpE* mutant for elastolytic protease. *Infect Immun* **68:** 5096–5106.

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988) Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70:** 191–197.

Keuntje, B., Masepohl, B., and Klipp, W. (1995) Expression of the *putA* gene encoding proline dehydrogenase from

Rhodobacter capsulatus is independent of NtrC regulation but requires an Lrp-like activator protein. *J Bacteriol* **177:** 6432–6439.

Kim, H.J., Lee, J.H., Rhee, J.E., Jeong, H.S., Choi, H.K., Chung, H.J., *et al.* (2002) Identification and functional analysis of the *putAP* genes encoding *Vibrio vulnificus* proline dehydrogenase and proline permease. *J Microbiol Biotechnol* **12:** 318–326.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lee, J.H., Park, N.Y., Lee, M.H., and Choi, S.H. (2003) Characterization of *Vibrio vulnificus putAP* operon, encoding proline dehydrogenase and proline permease, and its differential expression in response to osmotic stress. *J Bacteriol* **185:** 3842–3852.

Linkous, D.A., and Oliver, J.D. (1999) Pathogenesis of *Vibrio vulnificus*. *FEMS Microbiol Lett* **174:** 207–214.

McLeod, S., and Johnson, R. (2001) Control of transcription by nucleoid proteins. *Curr Opin Microbiol* **4:** 152–159.

Maloy, S.R. (1987) The proline utilization operon. In Escherichia coli *and* Salmonella typhimurium*: Cellular and Molecular Biology*. Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umbarger, H.E. (eds). Washington, DC: American Society for Microbiology Press, pp. 1513–1519.

Maloy, S., and Roth, J. (1983) Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put*:: Mud (Ap *lac*) operon fusion. *J Bacteriol* **154:** 561–568.

Maloy, S.R., and Stewart, V. (1993) Autogenous regulation of gene expression. *J Bacteriol* **175:** 307–316.

Miller, V.L., and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170:** 2575–2583.

Muro-Pastor, A.M., Ostrovsky, P., and Maloy, S. (1997) Regulation of gene expression by repressor localization: biochemical evidence that membrane and DNA binding by the PutA protein are mutually exclusive. *J Bacteriol* **179:** 2788–2791.

Oka, A., Sugisaki, H., and Takanami, M. (1981) Nucleotide sequence of the kanamycin resistance transposon Tn903. *J Mol Biol* **147:** 217–226.

Ostrovsky, P., and Maloy, S. (1993) PutA protein, a membrane-associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator. *Proc Natl Acad Sci USA* **90:** 4295–4298.

Ostrovsky, P., O'Brien, K., and Maloy, S. (1991) Regulation of proline utilization in *Salmonella typhimurium*: a membrane-associated dehydrogenase binds DNA *in vitro*. *J Bacteriol* **173:** 211–219.

Record, M.T., Jr, Courtenay, E.S., Cayley, D.S., and Guttman, H.J. (1998) Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem Sci* **23:** 143–148.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Strom, M.S., and Paranjpye, R.N. (2000) Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes Infect* **2:** 177–188.

Ventosa, A., Nieto, J.J., and Oren, A. (1998) Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62:** 504–544.

Vilchez, S., Molina, L., Ramos, C., and Ramos, J.L. (2000) Proline catabolism by *Pseudomonas putida*: cloning, characterization, and expression of the *put* genes in the presence of root exudates. *J Bacteriol* **182:** 91–99.