J. Microbiol. Biotechnol. (2008), **18**(11), 1755–1761
doi: 10.4014/jmb.0800.121
First published online 24 July 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Activation of the *Vibrio vulnificus cadBA* Operon by Leucine-Responsive Regulatory Protein is Mediated by CadC

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Received: February 11, 2008 / Accepted: April 8, 2008

The present study revealed that Lrp, a leucine-responsive regulatory protein, is involved in the regulation of *cadBA* transcription through activation of P_{cadBA}. The influence of Lrp on P_{cadBA} was mediated by CadC, and thereby, CadC was able to compensate for the lack of Lrp in the activation of P_{cadBA}. Western blot analyses and EMSA demonstrated that the cellular level of CadC was not significantly affected by Lrp, and that Lrp exerted its effect by directly binding to P_{cadBA}. These combined results suggested that CadC and Lrp function cooperatively to activate the P_{cadBA} rather than sequentially in a regulatory cascade.

Keywords: *Vibrio vulnificus*, *cadBA*, CadC, Lrp

For development of a disease, survival and multiplication are clearly the priorities of the infecting microorganisms. Therefore, it has been generally accepted that virulence factors include all those factors contributing to survival and multiplication on or within the host as well as to disease [14]. *Vibrio vulnificus* is an opportunistic Gram-negative pathogen that commonly contaminates various raw seafoods, and is the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia. [5, 13, 27]. Like many other pathogenic bacteria, *V. vulnificus* occurs in various environments with different stresses; it naturally inhabits coastal seawaters, contaminates shellfish, and infects the human body. This indicates that the pathogen has to overcome numerous stresses, imposed not only by natural ecosystems and currently available control practices, but also by the human immune defense system, to ensure developing illness.

The multifaceted nature of the stresses indicates that survival of the pathogenic bacteria is a complex phenotype and typically involves the products of many genes [14]. Most of these gene products act cooperatively to obtain maximum effectiveness in the survival, and their expression is coordinately controlled by a common global regulatory system in response to environmental signals [15]. Leucine-responsive regulatory protein (Lrp) is a transcription regulator that controls the expression of a number of genes involved in stress tolerance properties of bacteria [17]. In *Escherichia coli*, Lrp controls more than 70 genes that are grouped together to reflect physiologically related functions. It has been suggested that *E. coli* uses Lrp to adapt for environments, ranging from easy life in the gut and stressed harsh realities of the outside world [17, 26].

A homolog of the *E. coli lrp* gene was previously identified from *V. vulnificus* and the amino acid sequence of the *V. vulnificus* Lrp (Vv-Lrp) was 92% to 98% identical to those of the Lrp from *E. coli*, *Salmonella* Typhimurium, and *V. cholerae* [7]. Mutational analyses revealed that Lrp contributes to the survival of *V. vulnificus* under various stresses such as acidic pH, and that their contribution is dependent on the growth phase [7]. However, until now, no molecular analysis of the role of the Lrp in the acid tolerance of *V. vulnificus* has been reported, and therefore, none of the genes potentially involved in the survival of the bacteria under acidic pH has been identified as an Lrp-regulated gene(s).

We recently demonstrated that the *V. vulnificus cadBA* genes encode a lysine/cadaverine antiporter and a lysine decarboxylase, whose activities together lead to the synthesis and excretion of cadaverine to counteract external acidification [22–24]. The *V. vulnificus cadBA* expression is activated by CadC in a pH-dependent manner, and CadC exerts its effects at a distance by directly binding to the upstream sequences of P_{cadBA} [23, 24]. Accordingly, in an effort to elucidate the role of Lrp in the acid tolerance of *V. vulnificus* at a molecular level, the current study was undertaken to examine the influence of the mutation of *lrp* on the activity of lysine decarboxylase and the P_{cadBA}

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promoter. The relationship between CadC and Lrp was also examined by determining the cellular level of CadC in an *lrp* background. The question of whether the global regulator Lrp directly or indirectly affects the expression of the acid tolerance gene *cadBA* was also examined in the present study.

The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, the *V. vulnificus* strains were grown in Luria-Bertani (LB) medium supplemented with 2.0% (w/v) NaCl (LBS). Lysine decarboxylase activity in the cells was determined according to procedures previously described [21, 22]. For analysis of *cadA* transcripts, the total cellular RNA from the *V. vulnificus* strains was isolated using a Trizol reagent kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's specifications. The DNA probe CADAP was prepared by labeling DNA fragments containing the *cadA* coding region with [α-^{32}P]dCTP as previously described [23, 25], and used for the Northern analyses of the *cadA* transcript. The cellular levels of Lrp and CadC were determined by Western blot analyses using the IgG fraction of rat anti-*V. vulnificus* CadC and rat anti-*V. vulnificus* Lrp serum, respectively, as previously described [8, 9, 12, 25].

Generation and Confirmation of the Δ*lrp* and Δ*lrp cadC* Mutants

Construction of the Δ*lrp* and mutant was carried out by the methods described previously [19, 20, 23], with slight modifications. Briefly, the *lrp* gene in pHS102 was inactivated *in vitro* by deletion of three-fifths (300-bp) of the *lrp* open reading frame using the primers LRP031, LRP032, LRP033, and LRP034, and by a linker scanning mutation method [10] (Fig. 1A, Table 1). The 0.2-kb Δ*lrp* from the resulting construct (pJR0320) was liberated and ligated with SalI-SphI-digested pDM4 [16], forming pJR0323. *E. coli* SM10 (λ*pir*), *tra* (containing pJR0323) and *V. vulnificus* ATCC29307 were used as a conjugal donor and recipient, respectively, to generate the Δ*lrp* mutant JR310 (Fig. 1A) [9, 11]. In a similar way, pJR0011, in which the *cadC* ORF was inactivated by insertion of the 1.2-kb *nptI* cassette [18], and JR310 as a recipient were used for construction of the Δ*lrp cadC* double-mutant JR313.

Construction of the mutants, in which wild-type genes were replaced with the disrupted allele, was confirmed by PCR, as shown in Fig. 1. The Δ*lrp* mutant JR310 was confirmed by PCR using a pair of primers, LRP003 and LRP004, as indicated in Table 2. The size of the PCR product from the mutant was approximately 0.3-kb smaller than that from wild type, indicating that the *lrp* gene in JR310 is successfully disrupted by deletion of its ORF (Fig. 1B). PCR analysis of genomic DNA from JR310 with primers CAD0001 and CAD0002 indicated that a 1.6-kb fragment was produced (Table 2), whereas genomic DNA from the Δ*lrp cadC* mutant JR313 resulted in an amplified DNA fragment approximately 2.8 kb in length. This 2.8-kb fragment was in agreement with the projected size of the DNA fragment containing the wild-type *cadC* (1.6 kb) and *nptI* gene (1.2 kb) (Fig. 1C).

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

Strain or plasmid	Relevant characteristics[a]	References
Bacterial strains		
E. coli		
SM10λ*pir*	*thi thr leu tonA lacY supE recA::*RP4-2-Tc::Mu λ*pir, oriT* of RP4; Km^r; conjugational donor	[4]
V. vulnificus		
ATCC29307	Clinical isolate	Laboratory Collection
JR201	ATCC29307, *cadC::nptI*; Km^r	[24]
JR310	ATCC29307, Δ*lrp*	This study
JR313	ATCC29307, Δ*lrp, cadC::nptI*; Km^r	This study
Plasmids		
pGEM-T easy	PCR product cloning vector; Ap^r	Promega
pDM4	R6K γ *ori*; *sacB*; suicide vector; *oriT* of RP4; Cm^r	[16]
pJR991	4.3-kb EcoRI fragment containing *cadB*, part of *cadC*, part of *cadA* in pGEM7zf(+); Ap^r	[22]
pJR0011	pCVD442 with *cadC::nptI*; Ap^r, Km^r	[24]
pJR0012	pRK415 with *cadC*; Tc^r	[24]
pHS103	pRK415 with *lrp*; Tc^r	[6]
pHS102	pUC18 with 2.1-kb PstI fragment containing *lrp*; Ap^r	[7]
pJR0320	pGEM-T easy with 0.2-kb Δ*lrp*; Ap^r	This study
pJR0323	pDM4 with 0.2-kb Δ*lrp*; Cm^r	This study

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

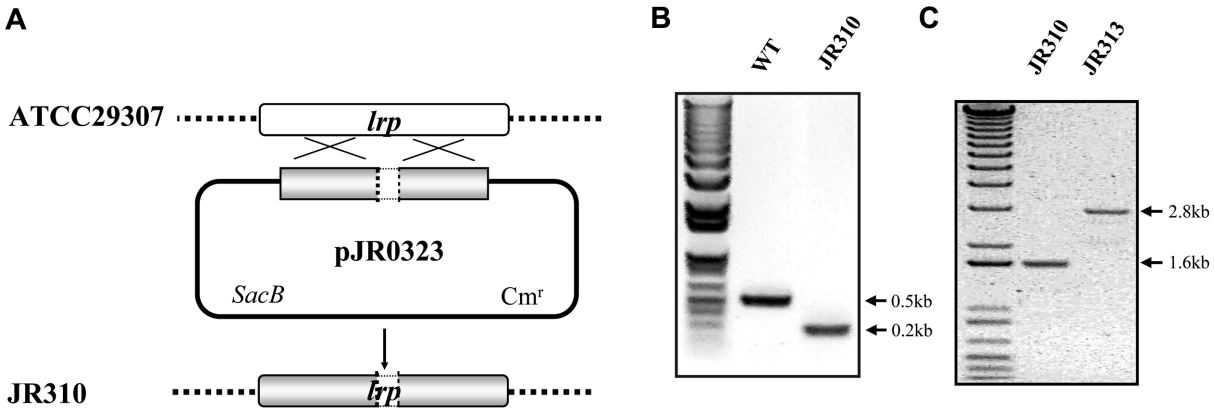

Fig. 1. Allelic exchange procedure and construction of the Δ*lrp* and Δ*lrp cadC* mutants.
A. Homologous recombination between the chromosomal *lrp* gene from wild-type ATCC29307 and pJR0323 led to deletion of the *lrp* gene and resulted in construction of the Δ*lrp* mutant JR310. In a similar way, the Δ*lrp cadC* double-mutant JR313 was constructed by homologous recombination between chromosomal *cadC* from JR310 and pJR0011, in which the *cadC* ORF was inactivated by insertion of the *nptI* cassette. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open boxes, the target *lrp* gene; large Xes represent genetic crossing over. Abbreviations; *sacB*, levansucrase gene; Cm^r, chloramphenicol resistant. **B**, **C**. PCR analysis of ATCC29307 and the Δ*lrp* or Δ*lrp cadC* mutants generated by allelic exchange. Molecular size markers (1-kb ladder; Invitrogen) appear on the left of the gels.

Effect of Mutation in the *lrp* Gene on Lysine Decarboxylase Activity

The *lrp* mutant JR310 produced approximately 0.4 unit of lysine decarboxylase, almost four-fold lower than that produced by the wild type (Fig. 2A). When pHS103 carrying recombinant *lrp* (Table 1) was reintroduced, the lysine decarboxylase activity in JR310 (pHS103) was restored to a level comparable to the wild-type level (Fig. 2A). Therefore, the decreased lysine decarboxylase activity in JR310 was proved to be due to functional inactivation of *lrp* rather than any polar effects on genes downstream of *lrp*. These results suggested that Lrp acts as a positive regulator in the expression of the *cadBA* operon of *V. vulnificus*.

The activities of the *cadBA* promoter, P_{cadBA}, in the wild type and isogenic mutants were compared using primer extension analyses. For the primer extension analysis, an end-labeled 24-base primer CAD9902 (Table 2) complementary to the coding region of the *cadB* was added to the RNA and then extended with SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen) as previously described [2, 8, 24, 25]. The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated from pJR991 (Table 1) with the same primer used for the primer extension. When compared with the wild type, a decreased reverse transcript was apparent with the RNA from the *lrp* mutant (Fig. 2B), indicating that the decreased level of transcription initiation of the P_{cadBA} plays the major, if not sole, role for the decreased lysine decarboxylase activity in the *lrp* mutant JR310. It was noted that the lysine decarboxylase and P_{cadBA} activities in the *cadC* mutant JR201 were significantly lower than those in the wild type and *lrp* mutant (Figs. 2A and 2B). This result

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Oligonucleotide sequence (5′→3′)[a]	Location[b]	Use
CAD9902	GCAATTAGGCCGATTTTCTTAGTA	66 to 89	Primer extension
CAD0001	CAACTGCAGCTAATGATTGGAATCTATTTTC	*cadC* on chromosome	Confirmation of Δ*lrp cadC* mutant
CAD0002	CAGGATCCGATAAGAAAATGAACCGCTCTCA	*cadC* on chromosome	Confirmation of Δ*lrp cadC* mutant
CAD0311	CGTTGATTTATGTATTGGGTG	−203 to −183	EMSA
CAD0312	GGCCGATTTTCTTAGTATCAGATG	61 to 84	EMSA
LRP003	GAATCTAGAGCAAGGAAGTAATAAGGTGGAAT	*lrp* on chromosome	Confirmation of Δ*lrp* mutant
LRP004	GAAGAATTCAAGGTGAAAGCAGCGATATAAGC	*lrp* on chromosome	Confirmation of Δ*lrp* mutant
LRP031	CCTTTGGAACGCCAATAATC	*lrp* on chromosome	Construction of *lrp* mutant
LRP032	CAGTGGATCCGACACGCTCTAAACATG	*lrp* on chromosome	Construction of *lrp* mutant
LRP033	TGTCGGATCCACTGATACTCTGCTTCG	*lrp* on chromosome	Construction of *lrp* mutant
LRP034	GAAAACCGTTCAGACGAGAAG	*lrp* on chromosome	Construction of *lrp* mutant

[a] Regions of oligonucleotide not complementary to the corresponding genes are underlined.
[b] Shown are the oligonucleotide positions, where +1 is the transcription start site of *cadBA*.

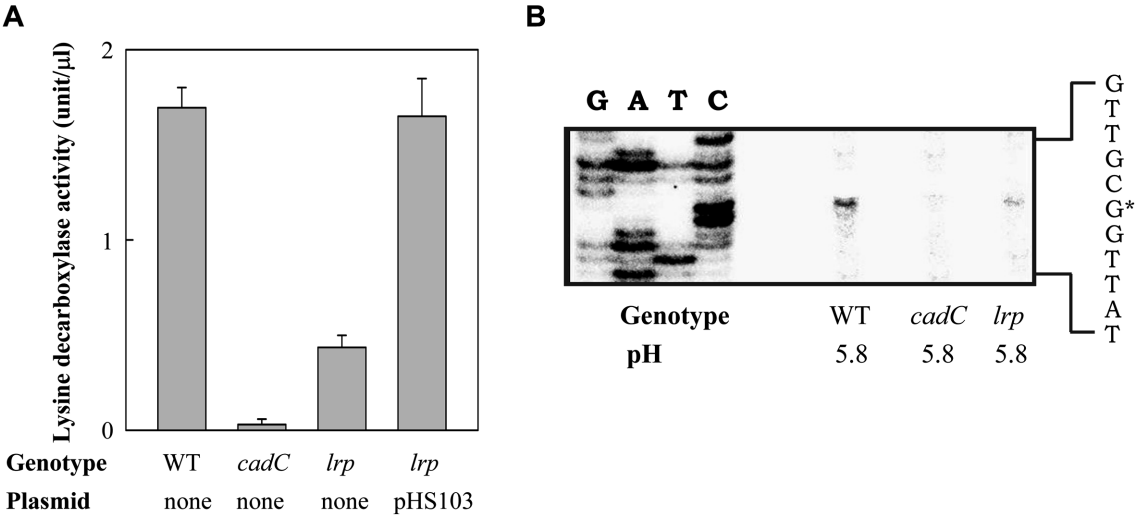

Fig. 2. Effect of mutation in the *lrp* gene on the lysine decarboxylase activity and transcription of P_{cadBA}.
A. Cultures were grown in LBS adjusted to pH 5.8. After 4 h of incubation, samples were removed and analyzed for lysine decarboxylase activity. Complementation of the mutant with a functional *lrp* (pHS103) is also presented as indicated. Error bars represent SEM. **B**. The P_{cadBA} activities were determined separately by primer extension of the RNA derived from the wild type and isogenic mutants, as indicated. Total RNA was prepared from each culture grown with LBS at pH 5.8. Lanes G, A, T, C represent the nucleotide sequencing ladders of pJR991. The asterisk indicates the site of the transcription start for P_{cadBA}. WT, wild type; *cadC*, *cadC* mutant; *lrp*, *lrp* mutant.

supports our previous observation that CadC activates the lysine decarboxylase expression in *V. vulnificus* [24], and also that the role of Lrp in activation of *cadBA* expression may be less significant than with that of CadC.

Effect of Lrp on P_{cadBA} Activity is Mediated Through CadC

The lysine decarboxylase activity and *cadA* transcript level in the Δ*lrp cadC* double mutant JR313 were indistinguishable from those in the *cadC* single mutant JR201 (Figs. 2A and 3), indicating that the additional inactivation of the *lrp* had no influence on the P_{cadBA} activity in the absence of CadC. Western blot analysis revealed that the Lrp level in the JR313 (pHS103) cells was relatively higher than that in the cells with the wild-type level of Lrp (Fig. 3C). Again, the lysine decarboxylase activity in the JR313 (pHS103) was almost identical to that in the *cadC* mutant JR201 (Figs. 2A and 3). From these results, it was apparent that a variation in the Lrp cellular level did not influence the P_{cadBA} activity unless CadC was also present. The results indicated that the effect of Lrp was mediated through CadC, suggesting that CadC is the primary regulator for the activation of P_{cadBA}.

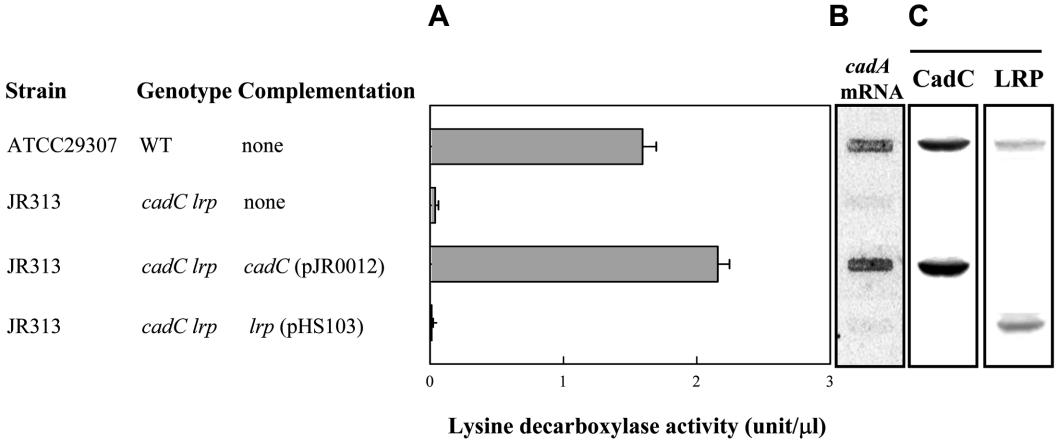

Fig. 3. The effect of Lrp on P_{cadBA} activity is mediated by CadC.
Samples were removed from cultures of the wild type and isogenic mutants, grown to OD_{600} of 0.7, and analyzed to determine lysine decarboxylase activity (**A**), *cadA* transcript (**B**), and CadC or Lrp levels (**C**). Details for determining the lysine decarboxylase activity, Northern slot-blot of the *cadA* transcript, and Western blot of Lrp and CadC are described in the text. For complementation tests, when 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 *cadC* (*i.e.*, on pJR0012) or *lrp* (*i.e.,* on pHS103), as indicated. Error bars represent SEM.

To determine if an increased amount of CadC would compensate for a lack of Lrp in the activation of P_{cadBA}, the *cadC* expression plasmid pJR0012 was introduced into the Δ*lrp cadC* double-mutant JR313. When *cadC* was induced by IPTG, the CadC cellular level in JR313 (pJR0012) was higher than that in the cells with the wild-type level of CadC (Fig. 3C). The lysine decarboxylase and the *cadA* transcript level in JR313 (pJR0012) were comparable to and even higher than those in the wild type (Fig. 3), indicating that CadC, when overproduced, was able to activate P_{cadBA} to the wild-type level in the absence of Lrp.

Lrp Cooperates with CadC by Directly Binding to P_{cadBA}

One interpretation of the ability of CadC to compensate for the lack of Lrp is that Lrp activates P_{cadBA} by enhancing either the cellular level or activity of CadC. For example, CadC and Lrp function sequentially in a regulatory cascade, where Lrp influences the accumulation of CadC, which in turn is directly responsible for the activation of P_{cadBA}. To test this possibility, the cellular levels of CadC were determined in the same amount of total protein isolated from both the wild type and its isogenic Δ*lrp* mutant (Fig. 4A). Western blot analysis revealed that the cellular level of CadC in the Δ*lrp* mutant was not significantly lower than that in the wild type (Fig. 4A), indicating that Lrp does not influence the accumulation of CadC. From this result, it is unlikely that Lrp indirectly activates the activity of P_{cadBA} by increasing the cellular level of CadC, which is required for P_{cadBA} activity. Despite the observation that the activation of P_{cadBA} by Lrp is mediated by CadC, the above result indicates that the influence of Lrp on P_{cadBA} is not due to the increase of CadC level in cells.

Another possibility that Lrp coactivates *cadBA* with CadC by directly binding to the P_{cadBA} promoter was examined by an electrophoretic mobility-shift assay (EMSA). For this, the 287-bp upstream region of the *cadBA*, extending from residues −203 to +84, was amplified by a PCR using ^{32}P-labeled CAD0311 and unlabeled CAD0312 as the primers (Table 2). The binding of Lrp to the labeled DNA and electrophoretic analysis of the DNA-Lrp complexes were performed as described previously [2, 8, 9, 25]. As shown in Fig. 4B, the addition of Lrp at a concentration of 1 μM resulted in a shift of the 287-bp DNA fragment carrying the P_{cadBA} regulatory region to a single band with a slower mobility. The binding of Lrp was also specific, because assays were performed in the presence of 1 μg of poly(dI-dC) as a nonspecific competitor. The result of the EMSA with Lrp indicated that Lrp binds to either a single binding site or to multiple binding sites with a comparable affinity. One possible explanation is that the binding of Lrp enhances the ability of CadC to activate P_{cadBA}. Consequently, it would appear that Lrp and CadC function cooperatively to activate P_{cadBA} activity rather than sequentially in a regulatory cascade.

We previously demonstrated that gene products of the *cadBA* contribute to the acid tolerance of *V. vulnificus*, and that their contribution is dependent on prior exposure of cells to a moderately acidic pH [22]. An open reading frame, *cadC*, was also identified upstream of the *cadBA*, and it was demonstrated that CadC acts as a positive regulator for the expression of the *cadBA* [23, 24]. It was noted that CadC activates the *cadBA* at a distance through direct binding to the P_{cadBA} promoter, and the CadC binding site is centered at −233.5 [24]. However, 233.5 bp upstream of the transcription start site is unusually distant for direct activation by a transcriptional regulatory protein [1]. For activation of *papBA* in *E. coli*, where CRP binding at −215.5 is one of the most exceptional distant bindings reported, CRP without Lrp provides little activation [28]. In this example, Lrp binds to multiple sites extended between the CRP binding and RNAP (RNA polymerase) binding. Furthermore, Lrp is proposed to bring CRP to contact C-terminal domain of the α-subunit (α-CTD) of RNAP by bending (forming a DNA loop) the promoter DNA [28].

Although other explanations are possible, we suggest that Lrp binds at specific sequences of the intervening

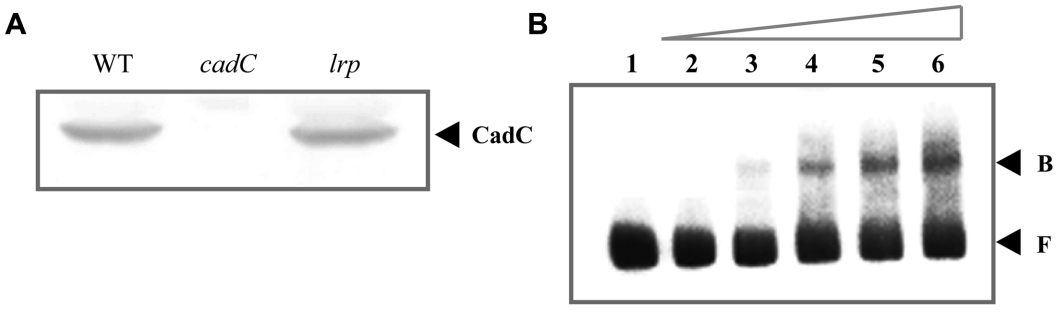

Fig. 4. Cellular levels of Lrp and CadC, and direct binding of Lrp to P_{cadBA}.
A. Cellular levels of CadC were examined by Western blot analyses as described in the text. **B**. Gel mobility-shift assay for binding of LRP to the *cadBA* regulatory region. A 287-bp DNA fragment of the upstream region of *cadBA* was radioactively labeled and then used as a DNA probe. The radiolabeled fragments (7 nM) were mixed with increasing amounts (0, 0.1, 0.5, 1, 1.5, and 2 μM in the first through sixth lanes, respectively) of LRP and then resolved on a 5% polyacrylamide gel. B, bound DNA; F, free DNA.

DNA between the CadC- and RNAP-binding sites of P_{cadBA}, thereby facilitating a protein-protein interaction between CadC and RNAP. Consistent with this, we previously demonstrated that these are three sequences with reasonable homologies to the Lrp binding consensus sequences in the intervening region [3, 24]. Experiments to clarify whether these regions really act as Lrp recognition sites are currently in progress.

Pathogenic bacteria have to constantly alter expression of many genes in response to ever-changing stresses in its growth environments [14]. Most of the genes and operons are members of a global regulatory network, and often subject to coordinate regulation. This coordinate regulation by global regulators would facilitate cooperation of the products of the genes, and would be crucial for the overall success in survival and multiplication of pathogenic bacteria on or within its host [14, 15]. Our previous report demonstrated that Lrp contributes to survival of *V. vulnificus* under numerous stresses such as acidic pH, cold temperature, and hyperosmolarity, indicating that the genes regulated by Lrp may be broad rather than specific [7]. However, until now, none of the experimental approaches, such as proteomic and transcriptome analysies, has yet been attempted for the extensive screening of the *V. vulnificus* genes that are regulated by Lrp. Nevertheless, identification of its target genes on a global scale would be of great interest, thus further characterizing the functions of the regulatory protein.

Acknowledgments

This study was supported by grants to S. H. C. from the MarineBio 21 Project, MOMAF, and the National Research Laboratory grant, KOSEF (R0A-2007-000-20039-0), ROK. We are indebted to H. S. Jeong for providing the plasmid pHS103.

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