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Identification of *Vibrio vulnificus lrp* and Its Influence on Survival Under Various Stresses

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Abstract An *lrp* gene encoding a leucine-responsive regulatory protein was identified from *Vibrio vulnificus*, and its role in the survival of the organism was assessed by analyzing the stress tolerance of the isogenic mutant, in which the *lrp* gene had been inactivated. The results demonstrated that Lrp contributes to the survival of *V. vulnificus*, and that their contribution is dependent on the phase of growth.

Key words: *V. vulnificus*, *lrp*, stress tolerance

Vibrio vulnificus is an opportunistic Gram-negative pathogen that commonly contaminates various raw seafood [11], and is the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia [7]. Predisposed individuals, with underlying immunocompromised conditions, liver damage, or excess levels of iron, who consume raw oysters, can die within days from sepsis. Even otherwise healthy people are susceptible to serious wound infections after contact with shellfish or water contaminated with *V. vulnificus* [1, 8]. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first signs of illness [1, 8].

The bacteria have evolved with elaborate protection systems to allow survival and/or growth during exposure to environmental stresses. Furthermore, pathogenic bacteria are highly adapted microorganisms with a survival strategy that requires multiplication on or within another living organism [14]. Pathogenic bacteria have to survive numerous stresses imposed upon not only by natural ecosystems and present control practices, but also by the human immune defense system, to ensure developing illness. This multifaceted nature of the stresses indicates that survival of the pathogenic bacteria is a complex phenotype and typically involves the products of many genes [12]. Most of the genes and operons are members of a global regulatory network, and often subject to coordinate regulation.

Global regulation is the concept that a single transcriptional regulator can control several distinct genes and operons in response to environmental signals. 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 [15]. In *E. 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 for adapting between the easy life in the gut and the stressed harsh realities of the outside world [15].

Like many other pathogenic bacteria, *V. vulnificus* occurs in various environments having different stresses; it naturally inhabits coastal seawaters, contaminates shellfish, and infects the human body. This indicates that the pathogen has to constantly alter expression of many genes in response to ever-changing stresses in its growth environments. However, until now, only a few studies have addressed the genes whose gene products contribute to stress tolerance of the pathogen [17]. Furthermore, no analysis of the effect of global regulators on the survival of the pathogen under various stresses has been reported. Therefore, in an effort to identify a global regulator involved in stress tolerance of *V. vulnificus*, current study identified and cloned an *lrp* gene encoding Lrp from *V. vulnificus*. A *V. vulnificus* null mutant, in which the *lrp* gene had been disrupted, was also constructed by allelic exchanges. After being challenged to the conditions, simulating current control practices used to suppress bacterial growth in raw seafood, such as low pH, hyperosmolarity and cold temperature, the survival of the mutant was compared with that of parental wildtype.

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

Strain or plasmid	Relevant characteristics[a]	References
Bacterial strains		
E. coli		
DH5α	*supE*44 Δ*lacU169* (*ϕ80 lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi-1 relA1*	Laboratory Collection
SY327λ*pir*	*Δ(lac pro) arg(*Am*) recA56 rpoB λpir;* Host for π-requiring plasmids	[13]
SM10λ*pir*	*thi thr leu tonA lacY supE recA::*RP4-2-Tc::Mu *λpir, oriT* of RP4, Km^r; conjugational donor	[13]
V. vulnificus		
ATCC29307	Clinical isolate	Laboratory Collection
HS02	ATCC29307, *lrp::nptI*	This study
Plasmids		
pUC18	Ap^r	Laboratory Collection
pLAFR3	IncP *ori*; cosmid vector; Tc^r	[18]
pUC4K	pUC4 with *nptI;* Ap^r, Km^r	Pharmacia
pCVD442	R6Kγ *ori*; *sacB, oriT* of RP4; Ap^r	[4]
pHS100	Cosmid library containing *lrp*; Tc^r	This study
pHS101	9.0-kb *Bam*HI fragment containing *lrp* cloned into pUC18; Ap^r	This study
pHS102	pUC18 with 2.1-kb *Pst*I fragment containing *lrp*; Ap^r	This study
pHS102-1	pHS102 with *nptI*; Ap^r, Km^r	This study
pHS102-2	pCVD442 with *lrp::nptI*; Ap^r, Km^r	This study

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

The bacterial strains and plasmids used in this study are listed in Table 1. Stress tolerance was assessed by measuring survivals in a challenged broth; M9 supplemented with 5.0% NaCl and incubated at 30°C (for osmotic tolerance) or M9 supplemented with 2.0% NaCl and incubated at 10°C (for cold stress). For acid tolerance, 10 mM sodium citrate buffer (pH 4.4) supplemented with 2.0% NaCl was used. Following inoculation into the challenged broths, samples were removed at appropriate intervals and plated in duplicate on LB [Luria-Bertani] supplemented with 2.0% NaCl as previously described [17]. The percentage of survivors was calculated relative to the CFU/ml as determined immediately after inoculation as 100%. Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations. The statistical significance of the difference among the *V. vulnificus* strains was evaluated using Students unpaired *t* test (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Significance was accepted at $p<0.05$.

Cloning and Sequencing Analysis of *V. vulnificus lrp*

To assess the role of *V. vulnificus* Lrp in stress tolerance, the *lrp* gene was cloned from strain ATCC29307. A DNA fragment internal to the *lrp* was then amplified from genomic DNA of *V. vulnificus* by a polymerase chain reaction (PCR) using a pair of oligonucleotide primers. The primers (the LRP01, 5'-GACCGTATAGATCGCAAT-AT-3', sense primer, and LRP02, 5'-GCTTCACTTCTTC-CATGAC-3', antisense primer) were designed using the *lrp* sequence of *V. cholerae* (GenBank accession number AE004266, www.ncbi.nlm.nih.gov) and synthesized (Takara, Seoul, Korea). Since the deduced amino acid sequence of the resulting PCR product, a 427-bp DNA fragment, revealed a 79% identity with that of *E. coli lrp*, the DNA

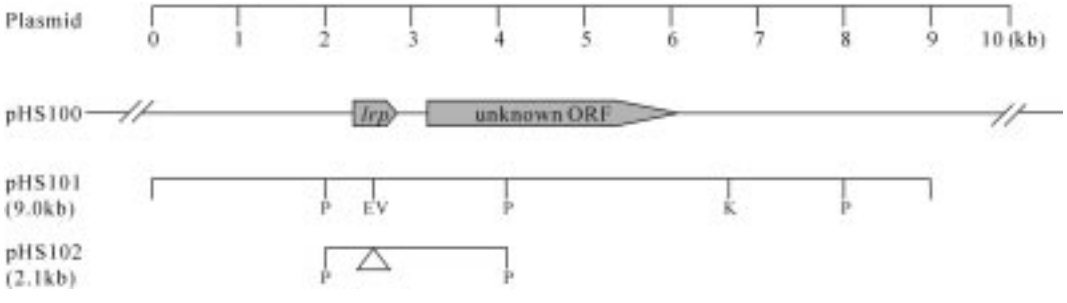

Fig. 1. Physical map of the *lrp* gene on the *V. vulnificus* ATCC29307 chromosome and plasmids used in this study.
Plasmid pHS101 was used to determine the nucleotide sequence of *lrp*. The shaded boxes represent the coding regions of *lrp* gene and unknown ORF, and thick lines represent chromosomal DNA, respectively. The region cloned in the plasmid pHS102-2, used for the construction of the *lrp::nptI* mutant, is depicted. The insertion positions of the *nptI* cassette are indicated by the open triangle. Abbreviations; EV, *Eco*RV; K, *Kpn*I; P, *Pst*I.

was labeled with [α-^{32}P] dCTP and named Lrp-P. To clone the full genes of the *lrp* gene, a cosmid library of *V. vulnificus* ATCC29307 constructed using pLAFR3 [18] was screened using Lrp-P as a probe. A colony showing a positive signal was isolated, and the cosmid DNA was purified and named pHS100 (Fig. 1). A 9.0-kb band from the cosmid DNA digested with *Bam*HI was purified and ligated into pUC18 (New England Biolabs) to result in pHS101 as shown in Fig. 1. The nucleotide sequence of the 9.0-kb DNA fragment in pHS101 was then determined by primer walking (Korea Basic Science Institute, Kwang-Ju, Korea). The nucleotide sequence of *lrp* from *V. vulnificus* ATCC29307 was deposited in the GenBank database under accession no. AY160773.

The nucleotide sequence revealed a coding region consisting of 468 nucleotides. A database search for nucleotide sequences similar to that of the coding region revealed three other *lrp* genes cloned from *E. coli*, *V. cholerae*, and *Salmonella typhimurium* strains with high levels of identity. The *lrp* from these bacteria was 79% to 84% identical in nucleotide sequence with the coding region in pHS101 (data not shown). This information proposed that the coding region was a homologue of *lrp* genes reported from other Enterobacteriaceae, and led us to name the coding region *lrp* to *V. vulnificus*. The amino acid sequence deduced from the *lrp* coding sequence revealed a protein, Lrp, composed of 155 amino acids with a theoretical molecular mass of 17,744 Da and PI of 5.88. The amino acid composition and molecular weight of this Lrp are quite similar to those of the Lrp from other Enterobacteriaceae. The amino acid sequence of the *V. vulnificus* Lrp was 92% to 98% identical to those of the Lrp from *E. coli*, *S. typhimurium*, and *V. cholerae*, and their identity appeared evenly throughout the whole proteins (Fig. 2, http//www.ebi.ac.uk/clustalw).

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V. vulnificus          ---------MDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR 41
V. cholerae            MVDSYKKPSKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR 50
S. typhimurium         MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR 50
E. coli                MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR 50
                                 ************************************

V. vulnificus          RLERQGYITGYTALLNPQYLDASLLVFVEITLNRGAPDVFEQFNAAVQKL 91
V. cholerae            RLERQGFITGYTALLNPQYLDASLLVFVEITLNRGAPDVFEQFNAAVQKL 100
S. typhimurium         RLERQGFIQGYTALLNPHYLDASLLVFVEITLNRGGPDVFEQFNAAVQKL 100
E. coli                RLERQGFIQGYTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNTAVQKL 100
                       ****** * ******** ***************** ******** *****

V. vulnificus          DDIQECHLVSGDFDYLLKTRVSDMGAYRKLLGDTLLRLPGVNDTRTYVVM 141
V. cholerae            DDIQECHLVSGDFDYLLKTRVSDMGAYRRLLGDTLLRLPGVNDTRTYVVM 150
S. typhimurium         BEIQECHLVSGDFDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVM 150
E. coli                BEIQECHLVSGDFDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVM 150
                         ******************** ** *** *** ******************

V. vulnificus          BEVKQTNQLVIKTR 155
V. cholerae            BEVKQTNQLVIKTR 164
S. typhimurium         BEVKQSNRLVIKTR 164
E. coli                BEVKQSNRLVIKTR 164
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Fig. 2. Sequence relatedness of Lrp of *V. vulnificus* and other bacteria.

Identical sequences are indicated with asterisks, and dashes represent missing sequences. Alignment was based on the amino acid sequences in the Genbank (NCBI) database and derived by the CLUSTRALW alignment program. (http://www.ch.embnet.org/software/ClustalW.html).

Generation and Confirmation of *V. vulnificus lrp* Mutant

The *lrp* gene in pHS102 that was constructed by ligation of a 2.1-kb *Pst*I fragment of pHS101 with pUC18 was inactivated *in vitro* by the insertion of *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin [16]. The 1.2-kb DNA fragment carrying *nptI* was isolated from pUC4K (Pharmacia, Piscataway, NJ, U.S.A.) and inserted into a unique *Eco*RV site present within the ORF of *lrp* in pHS102. The 3.3-kb *lrp*::*nptI* cartridge from the resulting construct (pHS102-1) was liberated and ligated with *Sma*I-digested pCVD442 [4], forming pHS102-2 (Fig. 1). To generate the *lrp::nptI* mutant in *V. vulnificus* by homologous recombination (Fig. 3A), *E. coli* SM10 λ*pir*, *tra* [13, 3] was transformed with pHS102-2, and used as a conjugal donor to *V. vulnificus* ATCC29307. The conjugation and isolation of the transconjugants were conducted using the methods previously described [6, 5]. A double crossover, in which each wildtype *lrp* gene was replaced with the *lrp::nptI* allele, was confirmed by a PCR using a pair of primers, LRP01 and LRP02 (Fig. 3B).

The PCR analysis of the genomic DNA from ATCC 29307 with primers LRP01 and LRP02 produced a 0.5-kb fragment (Fig. 3B), whereas the genomic DNA from the *lrp::nptI* mutant resulted in an amplified DNA fragment of approximately 1.7-kb in length. The 1.7-kb fragment was in agreement with the projected size of the DNA fragment containing the wildtype *lrp* (0.5-kb) and the *nptI* gene (1.2-kb). To determine the stability of the insertional mutation, strain HS02 was grown overnight without kanamycin selection. The inserted *nptI* DNA was stably maintained, evidenced by the maintenance of kanamycin resistance and by the generation of the appropriate-sized DNA fragment by PCR (data not shown). The *V. vulnificus lrp*

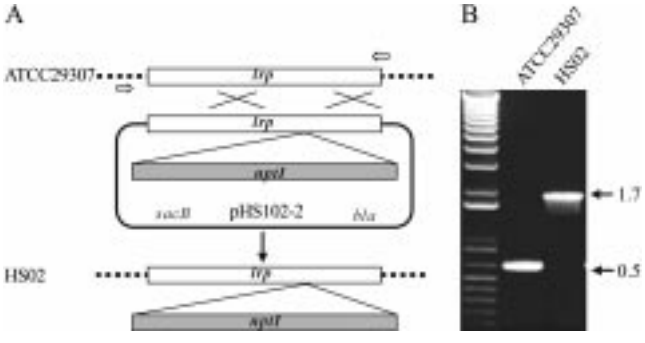

Fig. 3. Allelic exchange procedure and construction of *lrp::nptI* isogenic mutant.

Double homologous recombination between strain ATCC29307 and plasmid PHS102-2 led to an interruption of *lrp* gene and resulted in construction of mutant HS02. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open boxes, the target *lrp* gene; shaded boxes, the *nptI* gene; large Xes represent genetic crossing over. Abbreviations; *sacB*, levansucrase gene; *bla*, β-lactamase gene (A). PCR analysis of ATCC29307 and isogenic mutant HS02 generated by allelic exchange. Molecular size markers (1-kb ladder, GIBCO-BRL, Gaithersburg, MD, U.S.A.) appear in the last lanes of the gel (B).

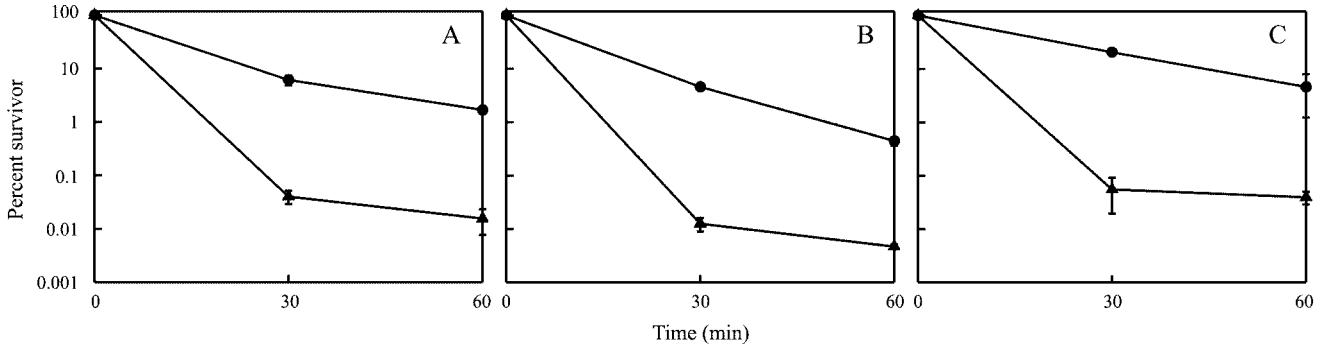

Fig. 4. Stress tolerance of log-phase ATCC29307 and isogenic *lrp* mutant.
Survival of log-phase *V. vulnificus* parent strain (ATCC 29307) (●) and *lrp::nptI* mutant (HS02) (▲). Both strains were challenged with acidic pH (A), low temperature (B), and hyperosmolarity (C) as described in the text. All points represent the mean from three independent trials. Error bars represent the standard errors.

mutant chosen for further analysis was named HS02 as shown in Fig. 3B.

Effect of *lrp* Mutation on Survival Under Various Stresses

The survival of log-phase cells (A_{600}=0.8) of the parent strain (ATCC29307) was significantly greater (p<0.05) than that of the *lrp::nptI* mutant (HS02), when challenged with acidic pH (pH 4.4; Fig. 4A). The parent strain decreased ca. 1.0 $\log_{10}$ CFU/ml (90%), while the mutant strain decreased ca. 4.0 $\log_{10}$ CFU/ml (99.99%) during a 60 min acid challenge. Similar to the results with the acid challenge, the survival of log-phase cells of the parent strain was significantly greater (p<0.05) than HS02, when challenged with either a low temperature (10°C) or hyperosmolarity (5% NaCl) (Figs. 4B and 4C). This indicated that log-phase cell of the *lrp* mutant was more sensitive to stresses than the wildtype, and that the Lrp plays an important role in the survival of *V. vulnificus*.

The survival of the cells grown to stationary phase (A_{600} =2.0) under stress challenges is shown in Fig. 5. The trend in survival of the stationary-phase cells (A_{600}=2.0) in an acidic pH was similar to that for log-phase cells. However, the survival of ATCC29307 and HS02 of stationary-phase cells increased significantly under an acid challenge. For ATCC29307, there was only a ca. 0.5 $\log_{10}$ CFU/ml decrease of stationary-phase cells compared to a ca. 1 $\log_{10}$ CFU/ml reduction with log-phase cells after a 60 min acid challenge. As observed for acid challenge, stationary-phase cells of both ATCC29307 and HS02 exhibited the slower rate of decline and had greater number of survivors after these stress challenges than log-phase cells. Additionally, compared to the results with log-phase cells, smaller differences in the survival of the parent strain and the *lrp* mutant were observed with stationary-phase cells (Fig. 5). Consistent with this, the survival of stationary-phase cells of the parent strain, ATCC29307, and HS02 in a hyperosmotic M9 broth was not significantly different, indicating that the role of Lrp in survival under high osmotic stress was not crucial for stationary-phase cells (Fig. 5C).

Consequently, it was apparent that the contribution of the *lrp* gene to the stress tolerance of *V. vulnificus* is substantial, but dependent on growth phases. It is also noteworthy that cells grown to stationary phase were more tolerant to stresses, regardless of the type of stresses challenged than log-phase cells. It has been previously reported that

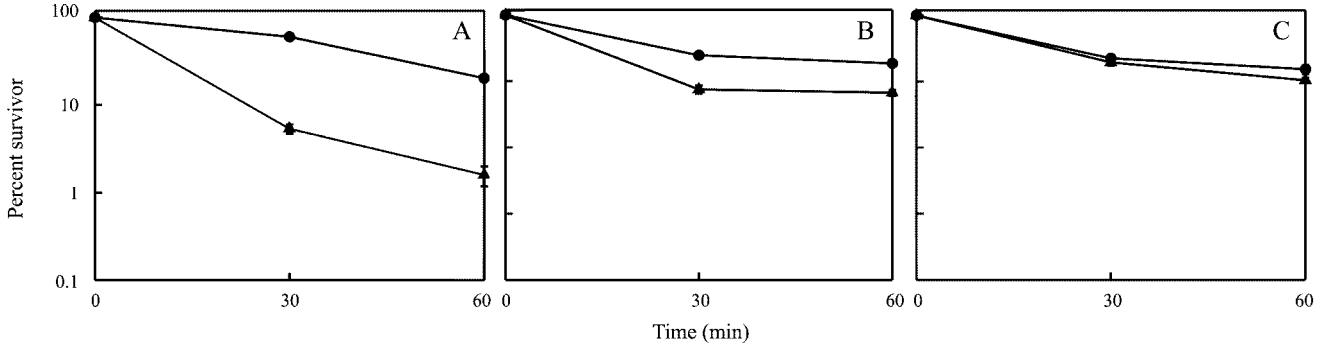

Fig. 5. Stress tolerance of stationary-phase ATCC29307 and isogenic *lrp* mutant.
Survival of stationary-phase *V. vulnificus* parent strain (ATCC 29307) (●) and *lrp::nptI* mutant (HS 02) (▲). Both strains were challenged with acidic pH (A), low temperature (B), and hyperosmolarity (C) as described in the text. All points represent the mean from three independent trials. Error bars represent the standard errors.

Enterobacteriaceae undergo a global modification of their gene expression pattern at the onset of the stationary phase [9, 10]. As a result, the bacteria acquire tolerance to a number of chemical and physical stresses, such as extreme temperatures, oxidative agents, hyperosmotic tension, and nutritional starvation. The stress tolerance of stationary-phase cells is multifactorial, and backup or redundant genes and factors contributing to the stress tolerance have often been identified. One well-known example is the RpoS regulon: In *E. coli*, the production of more than 40 proteins is associated with stationary-phase tolerance to stresses and encoded by genes of the RpoS regulon, and these stationary genes are regulated by RpoS (σ^S or σ^{38}), a stationary phase-specific sigma factor [19, 2]. Although other explanations are possible, the smaller differences in stress tolerance between the *lrp* mutant and the parent strain grown to stationary phase could be related to the presence of these other stationary genes contributing to the survival of *V. vulnificus*.

Until now, the role of the Lrp either in stress tolerance or in the survival of *V. vulnificus* has never been established. From the point of bacterial pathogenesis, the finding that Lrp contributes to the survival of *V. vulnificus* under various stresses would be of a great interest. When the pathogen invades a human body, the scarcity of specific nutrients and increased stresses imposed upon by the host immune defense system would be encountered. Therefore, the bacteria must survive these stresses in order to multiply and finally result in local damage and systemic disease. This survival often involves coordinate expression of sets of assorted genes [14] and many of these genes are probably regulated by Lrp.

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