J. Microbiol. Biotechnol. (2005), **15**(6), 1197–1206

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

luxS and *smcR* Quorum-Sensing System of *Vibrio vulnificus* as an Important Factor for *In Vivo* Survival

SHIN, NA-RI, CHANG-HO BAEK[1], DEOG-YONG LEE, YOUNG-WOOK CHO, DAE-KYUN PARK[1], KO-EUN LEE[1], KUN-SOO KIM[1], AND HAN-SANG YOO*

Department of Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea
[1]Department of Life Science, College of Natural Science, Sogang University, Seoul 121-742, Korea

Received: December 28, 2004
Accepted: April 4, 2005

Abstract *Vibrio vulnificus* is an opportunistic pathogen that causes a septicemia and expresses numerous virulence factors, in which *luxS* and *smcR* are genes encoding for components responsible for quorum-sensing regulation. In the present study, null mutants were constructed with lesions in each or both of these two genes from the *V. vulnificus* VvΔZ strain, which is a *lacZ*$^-$ and chloramphenicol/streptomycin-resistant derivative of the wild-type ATCC29307 strain, and their phenotypes related to virulence were compared with those of the parental cells. LD_{50} and histopathological findings of *luxS*-, *smcR*-, or *luxS- smcR*- deficient mutant were not different from those of the parent strain, a *lacZ*-deficient streptomycin-resistant strain in mice. However, time of death in mice was delayed, and numbers of bacteria survived in bloodstream after intraperitoneal injection in mice were decreased by mutation, especially *luxS* and *smcR* double mutant (VvSRΔZSR). These phenomena were supported by increased serum sensitivity and delayed bacterial proliferation in both murine blood and iron-restricted medium. These results suggest that the *luxS* and *luxR* homologous genes in *V. vulnificus* could play a role in bacterial survival in host by enhancing proliferation and adjusting to changed environment.

Key words: *Vibrio vulnificus*, *luxS*, *smcR*

Quorum-sensing is an intercellular signaling mechanism in which bacteria monitor their own or other bacterial population density by recognizing local concentration of chemical molecules, referred to as autoinducers, produced by the same bacterial species. The signal molecules regulate the expression of target genes at a minimal threshold stimulatory concentration.

*Corresponding author
Phone: 82-2-880-1263; Fax: 82-2-874-2738;
E-mail: yoohs@snu.ac.kr

Quorum-sensing circuits have been identified in over 30 species of Gram-negative bacteria. Most of the circuits resemble the canonical quorum-sensing of the symbiotic bacterium *Vibrio fischeri*, containing two regulatory proteins known as LuxI and LuxR [26]. LuxI is responsible for the production of autoinducer such as *N*-acylated homoserine lactones (AHLs), and the protein of LuxR family has two different domains, one for binding AHL and the other for binding DNA, which binds autoinducer and regulates transcription of target genes, respectively. LuxS, the AI-2 synthetase, is related with protease and hemolysin production [15]. LuxS cleaves S-ribosylhomocysteine to produce a precursor of AI-2, 4,5-dihydroxy-2,3-pentanedione [15, 36]. In a number of bacteria, quorum-sensing has been reported to control various processes related to interaction with host organisms including bioluminescence [8, 9], virulence [2, 18, 42], biofilm formation [6], antibiotic synthesis [1], plasmid conjugal transfer [31, 46], poly-β-hydroxybutyrates content [16] and siderophore production [41].

V. vulnificus, an opportunistic pathogen, is a Gram-negative estuarine bacterium that causes severe necrotizing wound infection [17] and primary septicemia in humans [27]. *V. vulnificus* infection is characterized by a high fatality rate, particularly in immunocompromised people and those with underlying conditions such as hemochromatosis, liver cirrhosis, and alcoholism [3–5, 25]. *V. vulnificus* produces several factors implicated in virulence and pathogenesis such as capsular polysaccharide, lipopolysaccharide (LPS), elastase, cytolysin, metalloprotease, siderophores, and phospholipase [10, 11, 34, 37, 39, 43, 45] and uses several genes for surviving or decreasing environmental stress such as *cadC*, *lrp*, and *rpoS* genes [12, 30, 33]. Like other *Vibrio* species, *V. vulnificus* has also been hypothesized to adopt quorum-sensing to regulate its pathogenesis. The quorum-sensing mechanism in *V. vulnificus*, however, has recently

begun to be elucidated and a few homologous genes have been reported. A homologue of the positive transcriptional regulator *luxR* of the *lux* operon in *V. harveyi*, named *smcR*, was identified in *V. vulnificus* [22, 38]. Our analysis of the genome sequence of *V. vulnificus* revealed a homologue of *luxS* of *V. harveyi*, which is responsible for the biosynthesis of the AI-2 molecule [36]. These imply that quorum-sensing regulation would be involved in pathogenesis of *V. vulnificus*. Therefore, further study was needed to determine the precise mechanisms associated with the gene regulation mediated by the quorum-sensing.

Based on the above knowledge, we investigated the pathological roles of these homologous genes that are possibly involved in the regulation of quorum-sensing. Our data strongly indicate that *luxS* and *smcR* are important factors *in vivo* for survival and proliferation of *V. vulnificus* at the early stage of infection.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains and plasmids used in this study are shown in Table 1. VvSR*ΔZ*, parent strain, is a *lacZ*$^-$ and spontaneously derived streptomycin-resistant mutant from VvAR, wild-type strain *V. vulnificus* ATCC29307. VvSR*ΔZS* and VvSR*ΔZR* are *luxS* and *smcR* deleted mutant from VvSR*ΔZ*, respectively. VvSR*ΔZSR* is a *luxS*$^-$ and *smcR*$^-$ double mutant strain constructed from VvSR*ΔZ*.

Mice and LD_{50}

Four to five week-old BALB/c mice (Daehan Biolink Co., EumSung, Korea) were used to determine the LD_{50} with *V. vulnificus* mutants. Experiments with mice were conducted following the guidelines on experimental animals set by Seoul National University, Korea.

Bacterial Culture

All strains were cultured in Luria-Bertani media (Difco, Detroit, MI, U.S.A.) supplemented with 2.5% NaCl (LBS) with vigorous shaking at 30°C or 37°C. For each experiment, bacteria were collected by centrifugation (10,000 ×*g* for 30 min), washed twice, and resuspended in phosphate-buffered saline (PBS, pH7.4). Colony forming units (CFU) per 1 ml of PBS of each *V. vulnificus* strain was determined on the basis of relationship between optical density at 600 nm and bacterial number confirmed by standard plate count method.

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains	Relevant characteristics	Sources or references
V. vulnificus strains		
VvAR	Wild-type strain ATCC29307	From Lee J. H. (Chonnam Nat'l Univ., Korea)
VvSR	Spontaneous Sm^R- mutant derived from VvAR,	This study
VvSR*ΔZ*	*lacZ*-null mutant derived from VvSR	This study
VvSR*ΔZS*	*luxS*-null mutant derived from VvSR*ΔZ*	This study
VvSR*ΔZR*	*smcR*-null mutant derived from VvSR*ΔZ*	This study
VvSR*ΔZSR*	*luxS* and *smcR* double-mutant derived from VvSR*ΔZ*	This study
E. coli strain		
S17-1:: λ-*pir*	*Pro* Res$^-$Mod$^+$Mob$^+$, Tp^r Sm^r	7
A. tumefaciens strain		
C58	Natural *lacZ*$^-$ strain	From Kim K. S. (Sogang University)
Plasmids		
pRK415	Cloning vector of RK2 origin, Tc^r	14
pRKVv12	pRK415 derivative cloned with *lacZ* from *V. vulnificus*	This study
pBSVE12-7	pBluescript SK- with 2.6-kb *Eco*RI-*Eco*RI fragment containing *lacZ* from *V. vulnificus*	This stidy
pBSVE12-7dSS	PBSVE12-7 with 935-bp *Sph*I/*Stu*I deletion in *lacZ* gene	This study
pKAS32	Suicide vector of R6K origin	37
pKAS32*ΔZ*	pKAS32 with 1.7-kb *Eco*RI-*Eco*RI fragment containing the deletion in 935-bp *Sph*I/*Stu*I fragment	This study
pRKVv*luxS*	PRK415 cloned with *luxS* of *V. vulnificus* ATCC29307	This study
PH103-21	Plasmid containing *smcR* of *V. vulnificus* ATCC29307 interrupted by insertion of *npt* (Km^R)	From Choi S. H. (Chunnam Nat'l Univ)
pKAS::RKO	pKAS32 with 2.4-kb *Eco*RI/*Xba*I fragment containing *smcR::npt*	This study

Abbreviation: Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; Tp, trimethoprim.

Construction of Genomic Library of *V. vulnificus* ATCC29307

Genomic library of *V. vulnificus* ATCC29307 was constructed into a broad-host-range IncP cloning vector pRK415, as described previously [14, 35].

Isolation of Sm-Resistant Derivatives. *V. vulnificus* ATCC29307 was grown on a solid Luria-Bertani medium containing 100 μg/ml of streptomycin (Sm), and spontaneous mutants grown on the medium were selected. One clone out of these mutants, showing growth indiscernible from that of wild-type cells, was isolated and named VvSR.

Construction of *lacZ*-Null Mutant. Genomic library clones of *V. vulnificus* ATCC29307 were introduced into *Agrobacterium tumefaciens* strain C58, a naturally *lacZ*$^-$ strain. Each transformant was patched on nutrient agar (NA; Difco, Sparks, MD, U.S.A.) containing X-gal (80 μg/ml), and clones showing blue-colored colonies were then isolated. The nucleotide sequence of one of the clones, pRKVv12, was determined, and an open reading frame (ORF) showing a significant similarity to *lacZ* in numerous bacteria was identified. A 2.6-kb *Eco*RI-internal fragment in *lacZ* was cloned from pRKVv12 into the unique *Eco*RI site of pBluescript SK$^-$ (Stratagene Co., Austin, Texas, U.S.A.) (Fig. 1A). Resulting plasmid, pBSVE12-7, was digested with *Sph*I and *Stu*I, and treated with T4 DNA polymerase and T4 DNA ligase to construct pBSVE12-7dSS, resulting in the deletion of the 935-bp *Sph*I/*Stu*I fragment from the 2.6-kb *Eco*RI fragment. The 1.7-kb *Eco*RI fragment from pBSVE12-7dSS was cloned into the unique *Eco*RI site of a suicide vector pKAS32 [39] to construct pKAS*ΔZ*. The plasmid was introduced into *V. vulnificus* strain VvSR by biparental mating using *E. coli* strain S17-1::λ-*pir* [7]. Exconjugants were selected on Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar (Difco) plate containing 100 μg/ml of ampicillin (Ap), and were streaked onto LB agar medium with 1 mg/ml of Sm and 40 μg/ml of X-gal. Sm-resistant (Sm^R) white colonies were tested for sensitivity to Ap (Ap^S). The *lacZ* deletions in these Sm^R-/Ap^S-white clones were further confirmed by PCR and Southern hybridization.

Construction of *luxS*-Deletion Mutant. A genomic library clone pRKVv*luxS* containing *luxS* of *V. vulnificus* ATCC29307 was isolated through colony hybridization with a probe obtained from PCR using the following primers: 5'-ATGCCATTATTAGATAG-3' and 5'-TTAATCCACTTTGAGCTC-3'. The PCR product comprised of a DNA fragment containing the *luxS*-coding region was confirmed by the nucleotide sequence analysis (data not shown). The 2.1-kb *Eco*RI/*Xba*I fragment containing the *luxS* gene was removed from pKAS*luxS* [Fig. 2A]. The 339-bp internal-

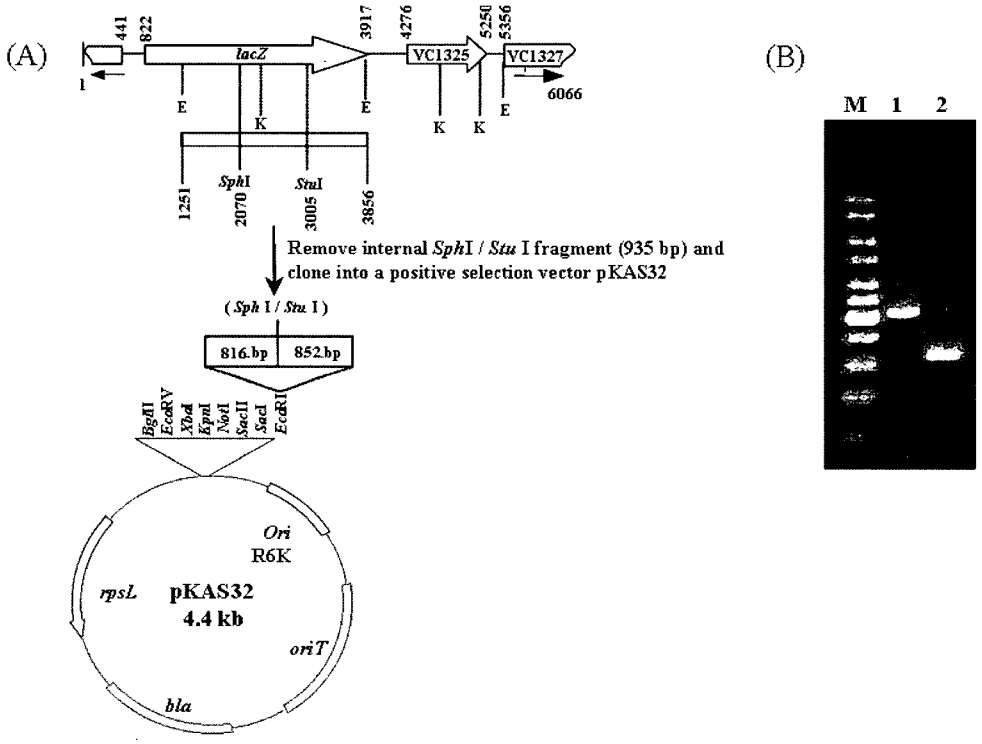

Fig. 1. Construction of *lacZ*-mutant by allelic exchange and its confirmation.

(A) Construction of pKAS32-derived suicide vector for the allelic exchange to generate the *lacZ*-null mutant. (B) PCR analysis of VvSR, which is an ATCC29307 derivative with resistance to streptomycin and rifampicin, and an isogenic mutant generated by allelic exchange. Lane M, molecular size markers (1-kb ladder, MBI); Lane 1, PCR product of VvSR; Lane 2, PCR product of *lacZ* deletion mutant (VvSRΔZ).

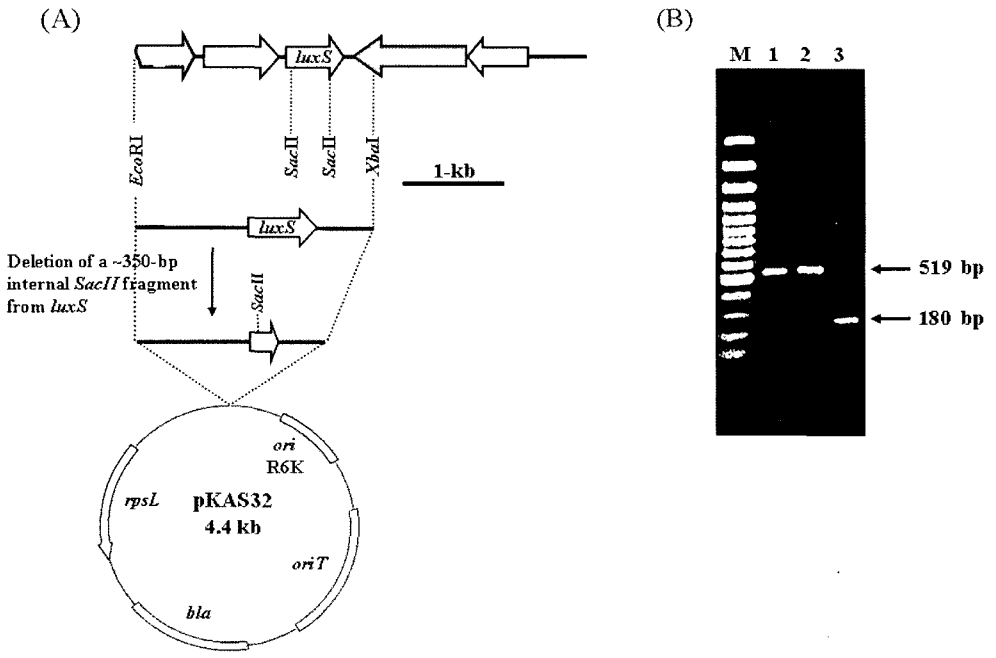

Fig. 2. Construction of a *luxS*-mutant by allelic exchange.
(A) Construction of pKAS32-derived suicide vector for the allelic exchange to generate the *luxS*-null mutant. (B) PCR analysis of VvSR, which is an ATCC29307 derivative with resistance to streptomycin and rifampicin, and isogenic mutants generated by allelic exchange. Lane M, molecular size markers (100-bp ladder, MBI); Lane 1, PCR product of VvSR; Lane 2, PCR product of *lacZ* deletion mutant (VvSRΔZ); Lane 3, PCR product of *lacZ-luxS-* double mutant (VvSRΔZS).

*Sac*II fragment of the *luxS* gene was removed from pKAS*luxS*. The resulting plasmid, pKASΔ*luxS*, was used for allelic exchange using pKAS32, as described above. The resulting construction was confirmed by PCR and Southern hybridization.

Construction of *smcR*-Deletion Mutant. The plasmid pH103-21 containing *smcR* of *V. vulnificus* ATCC29307 that was interrupted by insertion of the *npt* (Km^R) gene was kindly provided by Professor S. H. Choi (Seoul National University, Korea). The 2.4-kb *Eco*RI/*Xba*I fragment from pH103-21 containing *smcR::npt* was cloned into pKAS32 [Fig. 3A]. The resulting plasmid pKAS::RKO was introduced into *V. vulnificus* strains VvSR*ΔZ* and VvSR*ΔZS* to obtain *smc*R^- derivatives, VvSR*ΔZR* and VvSR*ΔZSR*, by allelic exchange.

LD_{50} in Mice

Ten-fold serially diluted bacterial suspension of the *V. vulnificus* strains were used for LD_{50} determination. Six mice per group were inoculated intraperitoneally. The number of dead mice was recorded 72 h after inoculation. Based on the mortality, LD_{50} were calculated as described by Reed and Muench [32]. This experiment was repeated three times.

Survival Rate of Mice Injected with *V. vulnificus*

The suspension of *V. vulnificus* (5.0×10^7 CFU/mouse) was intraperitoneally inoculated into mice. Groups of six mice were used for this experiment. Survival rate was recorded as a percentage of the number of live mice to the number of inoculated mice at 1-h intervals after injection. This experiment was repeated three times under the same condition.

Number of Bacteria in Blood after Intraperitoneal Injection

This was carried out as described by Shao and Hor [37]. Briefly, employing groups of twenty-five mice, 1.0×10^6 CFU of each *V. vulnificus* strain was simultaneously inoculated into the peritoneal cavity of mice, and four mice per group were randomly selected every hour after inoculation. The number of bacteria in blood collected from intraorbital venus of mice was estimated by the standard plate count method at 1-h intervals after inoculation. This experiment was repeated twice under the same condition.

Cytokine Concentration in Blood

Sera were collected from mice at 1-h intervals after intraperitoneal inoculation of 1.0×10^6 CFU of each strain tested. Concentration of TNFα, IL-1β, and IL-6 were measured by colorimetric sandwich ELISA (Endogen, Rockford, IL, U.S.A.) according to the manufacturer's protocol.

Assay for Bacterial Growth in Murine Blood

Bacterial growth in murine blood was measured as described by Shao *et al.* [37]. Briefly, each whole blood from three

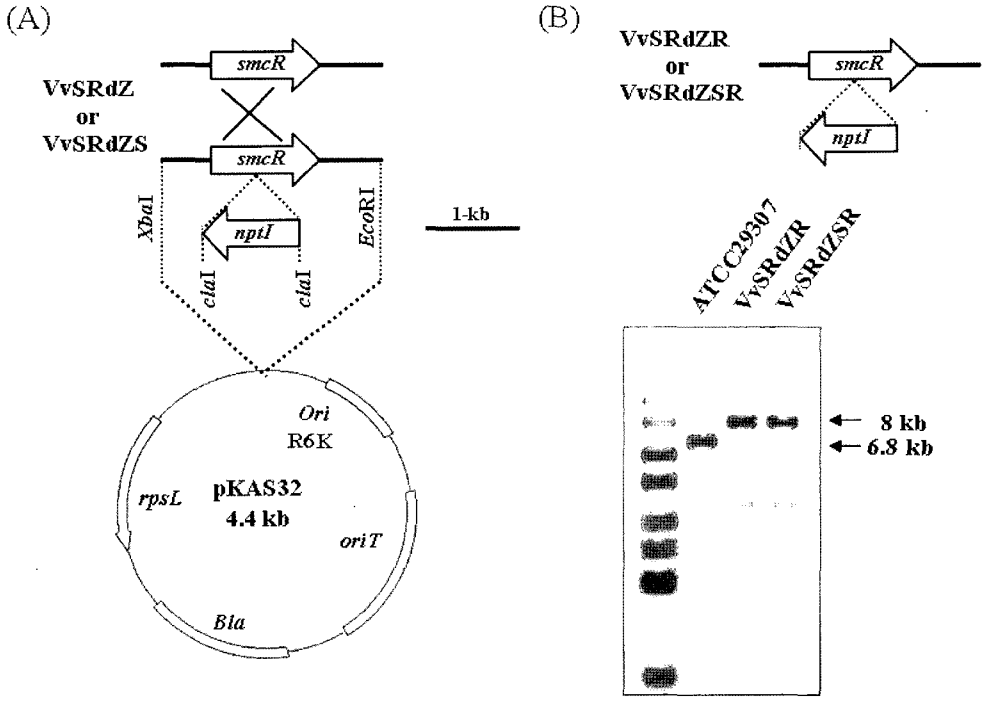

Fig. 3. Construction of a *smcR*-mutant by allelic exchange.
(A) Construction of pKAS32-derived suicide vector for the allelic exchange to generate the *smcR*-null mutant. (B) Southern blot analysis of strain ATCC29307 and isogenic mutants generated by allelic exchange. Genomic DNAs from strain ATCC29307, VvSRdZR, and VvSRdZSR were digested with *Sac*I and hybridized to a DNA probe consisting of the *smcR* coding sequence.

mice was collected and treated with heparin. Seventy μl of the bacterial suspension in PBS (5.0×10^1 CFU/ml) was mixed with 130 μl of each heparinized murine whole blood, and then incubated at 37°C with shaking.

The number of bacteria proliferated in the blood was measured at 2, 4, and 6 h after incubation by the standard plate count method. This experiment was repeated twice under the same condition.

Bacterial Proliferation in Iron-Restricted State

One-hundred μl of 50-fold diluted bacteria in stationary phase were inoculated into 20 ml of LBS with or without 100 μM dipyridyl (Sigma), and then incubated at 37°C with shaking. After taken every hour up to stationary phase, the cultures were washed and suspended with PBS. Optical density of the suspension was measured at 600 nm. Samples in triplicate were used, and the experiment was repeated three times.

Serum Sensitivity

Serum sensitivity of the bacteria was determined as described by Paranjpye *et al.* [28]. Briefly, bacterial cells washed with physiological saline were diluted fifty fold of the original culture. Two-hundred μl aliquots of diluted bacterial cells were mixed with 0.8 ml of human serum (Sigma, St. Louis, MO, U.S.A.) with or without heat inactivation (56°C, 30 min), and they were incubated at 37°C for 1 h. The bacterial number of each strain after incubation was measured by the standard plate count method, using the bacterial number of each strain before incubation as a control. Sensitivity was expressed as a ratio of an initial bacterial number before incubation to that of recovered after 1 h of incubation. This was repeated three times under the same condition.

Statistical Analysis

Geometric means of the numbers of bacteria from each group were calculated, and the significance of differences among means of the groups was determined by an unpaired *t*-test. Probability values at $P<0.05$ were considered as significant. For measurement of virulence and quantification of cytokines in sera, all data were given as means±standard deviations (SD).

RESULTS

Isolation of *luxS*-, *smcR*-, or *luxS*- *smcR*- Deficient *V. vulnificus* Mutants

luxS-, *smcR*-, or *luxS*- *smcR*- deficient mutants were constructed from *V. vulnificus* ATCC29307 to elucidate the roles of quorum-sensing genes on the regulation of virulence in *V. vulnificus*. VvSRs, spontaneous mutants grown on the medium containing streptomycin and rifampicin, were isolated,

and one of them was used as a recipient in conjugation to construct various mutants. A suicide vector pKAS32 containing *LacZ* in the deletion of the 935-bp fragment was introduced into VvSR by biparental mating using the *E. coli* strain, and the *lacZ*-null mutant derived from VvSR was named VvSR*ΔZ*.

The Δ*lacZ* mutant was confirmed by PCR (Fig. 1B) and Southern hybridization (data not shown). The *lacZ* mutant was used as a selection marker in the consecutive experiment in this study. The *lacZ* sequence was registered in the GenBank database with the accession number AY028965.

A suicide vector carrying the *luxS* gene with a 339-bp deletion was used to isolate the *ΔluxS* mutant through an allelic exchange technique (Fig. 2).

The nucleotide sequence of the *luxS*-coding region in pRKVv*luxS* was determined and deposited in the GenBank database under the accession number AF401230. The *luxS*-deletion derivative of strain VvSR*ΔZ* was called VvSR*ΔZS*. VvSR*ΔZR* and VvSR*ΔZSR* were constructed by introducing pKAS::RKO, the plasmid in which *smcR* was interrupted by insertion of the *npt* (Km^R) gene, into VvSR*ΔZ* and VvSR*ΔZS*, respectively, by allelic exchanges (Fig. 3).

LD_{50} in Mice.

LD_{50} of *luxS*-mutant was the highest among mutants, even though there was no statistical significance. In addition, LD_{50} of *V. vulnificus* strains against mice was not different between the genotypes (data not shown).

Survival Rate of Mice Against *V. vulnificus* Strains

Survival patterns of mice infected with 5.0×10^7 CFU of wild-type or mutants are shown in Fig. 4. Differences in survival rate according to time were observed among the mutants, even though all infected mice were dead in 8 h after injection. More than 80% of mice survived in the group

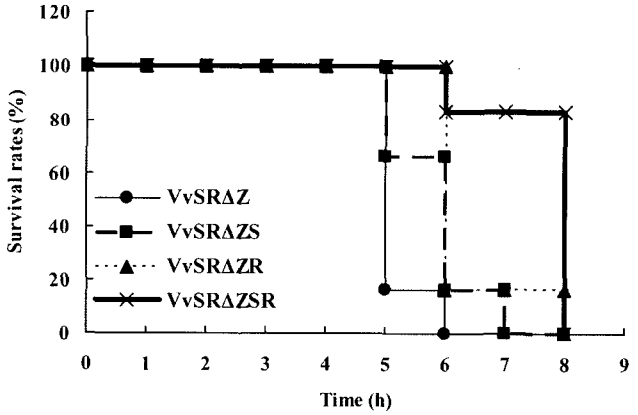

Fig. 4. Survival rate of mice injected with 5.0×10^7 CFU/mouse of *Vibrio vulnificus* in time-course.
Survival rate was expressed as a percentage of survived mice to inoculated mice at 1-h intervals after injection.

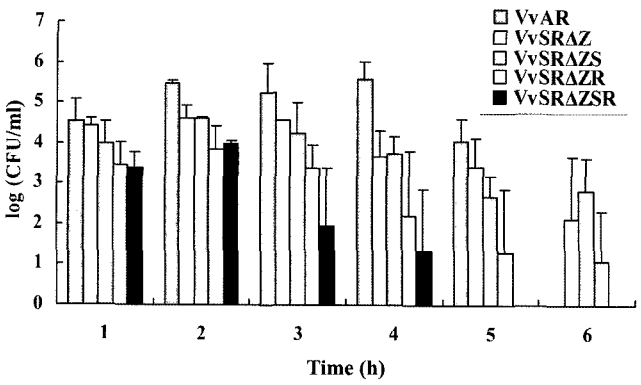

Fig. 5. Invasion of *Vibrio vulnificus* strains into bloodstream and bacterial survival in blood after intraperitoneal injection.

challenged with *luxS⁻smcR⁻* double mutant ((VvSR*ΔZSR*) at 7 h post injection, whereas all mice died in the group challenged with a *lacZ*-null mutant containing *luxS* and *smcR* (VvSR*ΔZ*) at the same time (Fig. 4). The simultaneous mutation of both *luxS* and *smcR* (VvSR*ΔZSR*) resulted in significant delay of the death of mice for 3 h, and *smcR* deleted mutant (VvSR*ΔZR*) showed less virulence than *luxS* deleted one (VvSR*ΔZS*).

Bacterial Invasion into Bloodstream and Bacterial Survival

Intravascular dissemination of *V. vulnificus* strains was assessed by counting viable bacteria in blood collected from the infraorbital vein after intraperitoneal injection. In all groups infected, the numbers of bacteria invading into bloodstream were not significantly different during the early stage of infection, even though wild-type (VvAR) showed the highest invading rate and began to decrease in 3 h after infection (Fig. 5). In particular, the number of *luxS⁻smcR⁻* double mutant (VvSR*ΔZSR*) in mouse bloodstream was decreased more than 100 folds, and only this mutant could not be detected from blood after 5 h. The result of *smcR⁻* mutant (VvSR*ΔZR*) showed significant decrease in bacterial number, whereas *luxS*-null mutant (VvSR*ΔZS*) was not significantly different from that of parent strain ($P<0.05$). All mice injected with wild-type (VvAR) died 6 h after infection. Clinical manifestations were also milder in mice injected with the *smcR⁻* mutant.

Cytokine Concentration in Blood

All proinflammatory cytokines tested were induced by parent strain, and their concentrations were 5 times higher than those induced by *luxS*(VvSR*ΔZS*), *smcR⁻* (VvSR*ΔZR*), or *luxS⁻smcR⁻* mutants (VvSR*ΔZSR*) (Fig. 6). In particular, TNFα was characteristically detected in mice injected with *luxS⁻smcR⁻* double mutant (Fig. 6a). IL-1β and IL-6 were detected in all groups, but had little difference among the groups (Figs. 6b and 6c).

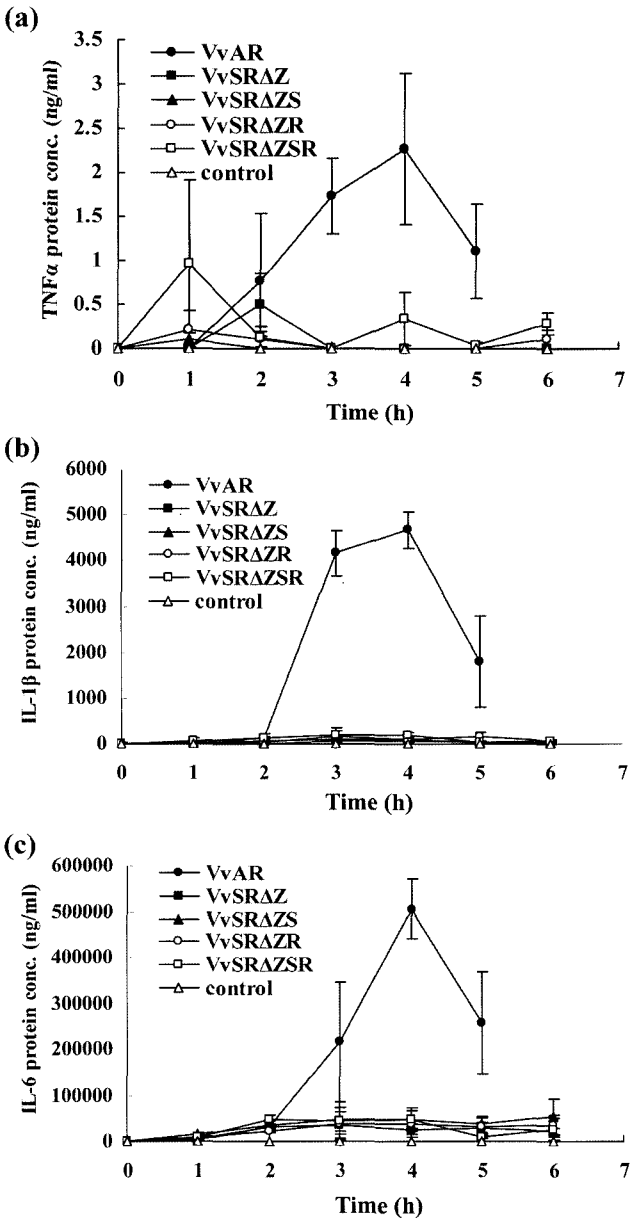

Fig. 6. Production of TNFα (a), IL-1β (b), and IL-6 (c) in murine blood after bacterial infection.
Sera were collected from mice infected with *Vibrio vulnificus* strains at 1-h intervals. Concentrations of cytokines were measured by colorimetric capture ELISAs.

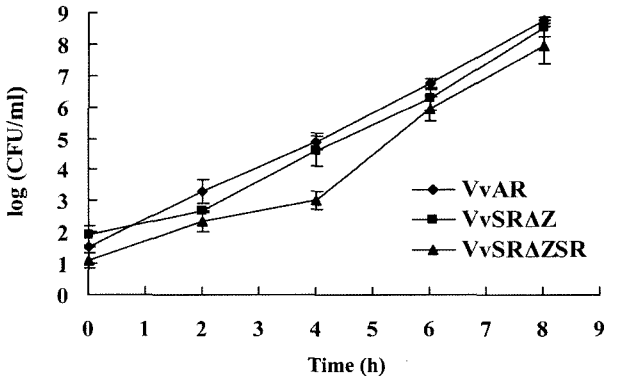

Fig. 7. Growth of *Vibrio vulnificus* strains in murine whole blood.
Bacterial strains were mixed with heparinized murine whole blood, and the numbers of bacteria survived were measured at 2-h intervals during incubation at 37°C by the standard plate count method.

Bacterial Growth in Blood

Bacterial growth in murine blood was measured by counting the number of bacteria at 2-h intervals after incubation *in vitro* (Fig. 7), and their proliferation in heparinized whole blood was time-dependent. It was notable that the growth rate of *luxS*$^-$ and *smcR*$^-$ double mutant was two-orders of magnitude lower between 2–4 h, and then returned to the level similar to that of parent strain, *luxS*$^+$*smcR*$^+$ strain, after 6 h.

Bacterial Proliferation in Iron-Restricted State

Iron was chelated using an iron chelator, dipyridyl, from LBS, and then the proliferative pattern of each strain in this medium was compared with that in an iron-rich medium. Bacterial growth of all strains tested was slow, and the time to reach the stationary phase was also delayed by iron restriction (Fig 8). In particular, the lag growth phase was prolonged by the double mutation of *luxS* and *smcR*. In the environment where the quantity of usable iron was restricted, the most remarkable decrease in proliferation was shown at 5 h postinoculation, and the optical density did not reach levels similar to other strains until 10 h after bacterial inoculation (Fig. 8b).

Sensitivities to Sera

Sensitivities to sera were measured by counting the number of surviving bacterial cells after 1 h of incubation with human sera with or without heat inactivation. In both *luxS*$^+$*smcR*$^+$ strain and *luxS*$^-$*smcR*$^-$ double mutant, the number of surviving bacterial cells was decreased by the incubation of bacteria with normal sera, compared with those incubated in heat-inactivated sera (Table 2), and the sensitivity of the *luxS*$^-$*smcR*$^-$ double mutant to sera was slightly but significantly higher than that of the *luxS*$^+$*smcR*$^+$ strain ($P<0.05$).

DISCUSSION

Unlike other *Vibrio* spp. [13, 22, 47], the quorum-sensing system in *V. vulnificus* is not yet clearly understood. However, it has been suggested that *V. vulnificus* adopts the quorum-sensing system in a manner similar to other *Vibrio* spp. such as *V. harveyi*, by identification of homologues of *luxS* and *luxR* [23]. High similarities in both nucleotide and amino acid sequences between *smcR* in *V. vulnificus* and *luxR* in *V. harveyi* suggest that these genes are

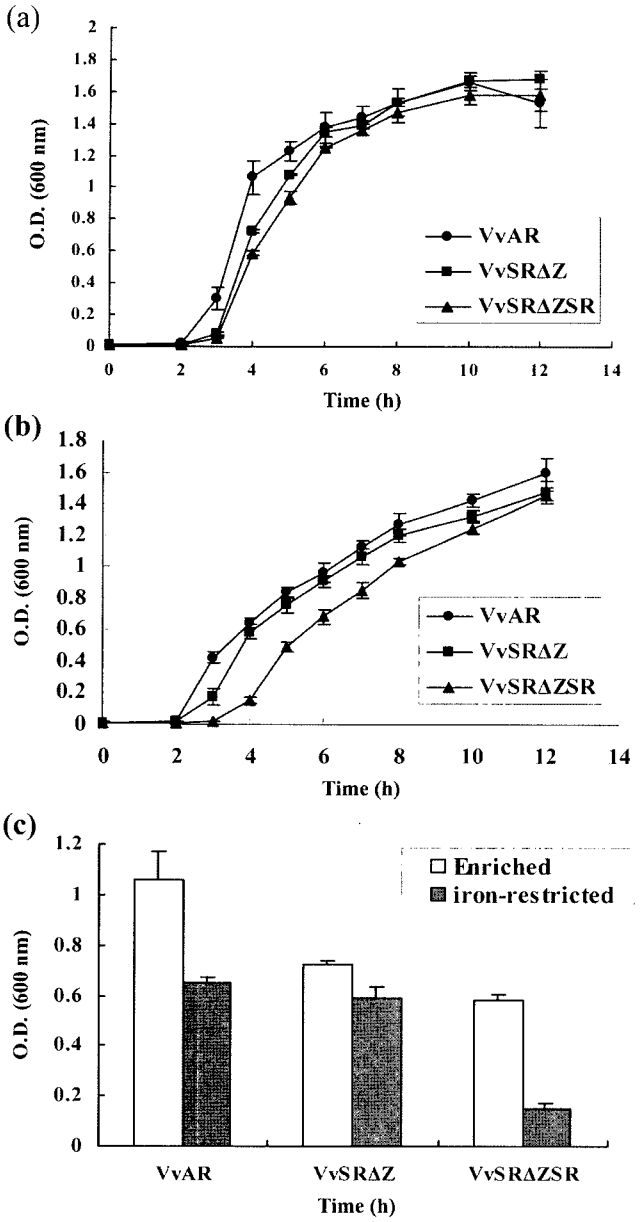

Fig. 8. Bacterial proliferation in time-course in LBS (a) or iron-restricted LBS medium (b) and comparison of optical density among the strains at 4 h of cultivation (c).
Proliferation was determined at 1- or 2-h intervals up to the stationary phase by measuring optical density at 600 nm. Proliferation of *luxS* and *smcR* double mutant was delayed in iron-restricted conditions.

functionally conserved, ancient, and widespread in marine *Vibrio* spp. [22]. It implies that the quorum-sensing circuit in *V. vulnificus* also plays a significant role in regulating expression of certain gene(s). Based on these hypotheses, the roles of *luxS* and *smcR* on phenotypes associated with virulence was investigated, and the data in this study suggest that *luxS* and *smcR* are important for pathogenesis in host, especially for the survival, adjustment, and proliferation of *V. vulnificus*.

Table 2. Serum sensitivity of *Vibrio vulnificus* strains VvSR*ΔZ* and VvSR*ΔZSR*.

Strain \ Serum	Normal	Heat-treated	Serum sensitivity
VvSR*ΔZ*	0.3398±0.0374	0.1522±0.0101	0.1876
VvSR*ΔZSR*	0.2542±0.07748	−0.0082±0.0967	0.2624

Mouse survival rates showed that both *luxS* and *smcR* mutations were able to delay mortality of mice (Fig. 4), in agreement with a recent report of Kim *et al.* [15] that *luxS* mutation could delay the time of death of mice. It suggests that both *luxS* and *smcR* could be involved in effective and rapid adjustment of *V. vulnificus* to sudden change of environment in the host. Nevertheless, the LD_{50} did not show a statistically significantly increase by *luxS* and *smcR* mutation (data not shown). Mortality in mice following *V. vulnificus* infection did not occur until the number of bacteria in blood reached more than 1.0×10^6 CFU/ml threshold. Furthermore, *luxS* and *smcR* mutation could not decrease bacterial invasion from the peritoneal cavity into the bloodstream (Fig. 5): In other words, the number of bacteria inoculated is the most critical factor for the LD_{50}. Therefore, the LD_{50} seemed to be not affected by *luxS*, *smcR* mutation.

The role of *luxS* and *smcR* on pathogenesis in mice was well established by the bacterial invasion and survival assay (Fig. 5). Our data suggest that *luxS* and *smcR* genes are associated with the survival of *V. vulnificus* in the bloodstream rather than invasion. This is supported by the shortest survival time of *luxS*$^-$*smcR*$^-$ double mutant in bloodstream after invasion (Fig. 5). In particular, *smcR* plays a more critical role in bacterial survival in host than *luxS* and this is supports our results on the survival rate of mice (Fig. 4) as well as a previous report on the importance of *smcR* in the adaptation of this organism to stress conditions such as starvation [23].

The importance of *luxS* and *smcR* in the early infection period was supported by the characteristic retardation of bacterial growth between 2–4 h in murine blood (Fig. 5) and increased sensitivity to sera, containing complements as representative bactericidal factors (Table 2). Once *V. vulnificus* infects the body, the bacteria enter into bloodstream and face the complement system and iron-restricted environment. At this point, *luxS* and *smcR* systems might help *V. vulnificus* to survive, throughly inducing rapid adjustment and proliferation. The data in the present study completely conform with that of the invasion assay and support the results of the invasion assay, showing rapid removal of *luxS*$^-$*smcR*$^-$ double mutant from host. Iron is particularly important in the pathogenesis of *V. vulnificus* infections. Wright *et al.* [44] showed positive correlation between the virulence of *V. vulnificus* and iron availability. Our data also showed that *luxS* and *smcR* double mutation remarkably delayed the time to reach logarithmic phase,

suggesting the roles of *luxS* and *smcR* in *in vivo* environment such as iron restriction.

In invasion assay, lethality to mice occurred only in the group injected with wild-type. It might have been due to acute progress of the infection: Acute septicemia and overproduction of cytokine might act as causes of death of mice following *V. vulnificus* infection, and it was identified by histopathological findings of typical septic changes characterized by increase of vascular permeability, neutrophil sequestration in lungs, and severe congestion (data not shown), which are similar to those reported by Park *et al.* [29]. Bradykinin generated by bacterial virulence factors acts as a universal mediator in inflammatory reaction [20]. On the other hand, generation of bradykinin in primary septic foci facilitates intravascular dissemination of bacteria and septicemia [19, 21]. These findings suggest that *luxS* and *smcR* might allow *V. vulnificus* to survive in bloodstream through activation of the bradykinin cascade. It is quite likely that *luxS* and *smcR* might promote bacterial survival by regulating other inflammatory reactions in host.

McDougald *et al.* [24] and Shao and Hor [38] reported that SmcR acts as a positive regulator of the metalloprotease gene. Roles of this protease were revealed in the bacterial growth and disease development, because the protease could facilitate iron acquisition by organisms by digesting heme proteins, such as transferrin and lactoferrin, and increasing vascular permeability and edema [24, 38]. However, deficiency of metalloprotease does not decrease both the bacterial invasion and growth in blood [36]. These reports together with our results infer that metalloprotease might be implicated in the pathogenesis by association with the function of *luxS* and *smcR* genes of *V. vulnificus* at the initial infection stage, although *smcR* activates expression of the metalloprotease gene.

In conclusion, this study demonstrated that the virulence of *V. vulnificus* in mice could be enhanced by *luxS*, and that *smcR* genes might be related to quorum-sensing through promoting bacterial adjustment, survival, and proliferation in the early stage of infection. It might also be involved in enhanced resistance to complement and in downregulation of initial immune response. However, to understand the precise mechanism of quorum-sensing in virulence of *V. vulnificus*, a larger number of mutants should be constructed, and other bacterial characteristics related to virulence should be investigated, using the newly constructed mutants, in further studies.

Acknowledgments

This study was supported by the Korean Science and Engineering Foundation, Brain Korea 21, and the Research Institute for Veterinary Science, Seoul National University, Korea.

References

1. Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. Salmond, G. S. Stewart, and P. Williams. 1992. N-3-oxohexanoyl-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. *Biochem. J.* **288:** 997–1004.
2. Beck van Bodman, S. and S. K. Farrand. 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an N-acylhomoserine lactone autoinducer. *J. Bacteriol.* **177:** 5000–5008.
3. Brennt, C. E., A. C. Wright, S. K. Dutta, and J. G. Morris, Jr. 1991. Growth of *Vibrio vulnificus* in serum from alcoholics: Association with high transferring iron saturation. *J. Infect. Dis.* **164:** 1030–1032.
4. Bullen, J. J., P. B. Spalding, C. G. Ward, and J. M. Gutteridge. 1991. Hemochromatosis, iron and septicemia caused by *Vibrio vulnificus*. *Arch. Intern. Med.* **151:** 1606–1609.
5. Chan, T. Y., D. P. Chow, K. C. Ng, K. W. Pan, and G. A. McBride. 1994. *Vibrio vulnificus* septicemia in a patient with liver cirrhosis. *Southeast Asian J. Trop. Med. Public Health* **25:** 215–216.
6. Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280:** 295–298.
7. De Lorenzo, V. and K. N. Timmis. 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235:** 386–405.
8. Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20:** 2444–2449.
9. Engebrecht, J., K. Nealson, and M. Silverman. 1983. Bacterial bioluminescence: Isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32:** 773–781.
10. Gray, L. D. and A. S. Kreger. 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. *Infect. Immun.* **48:** 62–72.
11. Hanne, L. F. and R. A. Finkelstein. 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. *Infect. Immun.* **36:** 209–214.
12. Jeong, H. S., J. E. Rhee, J. H. Lee, H. K. Choi, D. I. Kim, M. H. Lee, S. J. Park, and S. H. Choi. 2003. Identification of *Vibrio vulnificus lrp* and its influence on survival under various stress. *J. Microbiol. Biotechnol.* **13:** 159–163.
13. Jobling, M. G. and R. K. Holmes. 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene hap, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol. Microbiol.* **26:** 1023–1034.
14. Keen, N. T., S. Takami, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70:** 191–197.
15. Kim, S. Y., S. E. Lee, Y. R. Kim, C. M. Kim, P. Y. Ryu, H. E. Choy, S. S. Chung, and J. H. Rhee. 2003. Regulation of

Vibrio vulnificus virulence by the LuxS quorum-sensing system. *Mol. Microbiol.* **48:** 1647–1664.
16. Kumamoto, K. S. and D. J. Vukich. 1998. Clinical infections of *Vibrio vulnificus*: A case report and review of the literature. *J. Emergency Med.* **16:** 61–66.
17. Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol. 1999. Quorum sensing in *Burkholderia cepacia*: Identification of the LuxRI homologs CepRI. *J. Bacteriol.* **181:** 748–756.
18. Maeda, H., T. Akaike, Y. Sakata, and K. Maruo. 1993. Role of bradykinin in microbial infection: Enhancement of septicemia by microbial proteases and kinin. *Agents Actions Suppl.* **42:** 159–165.
19. Maeda, H. and T. Yamamoto. 1996. Pathogenic mechanisms induced by microbial proteases in microbial infection. *Biol. Chem. Hoppe-Seyler* **377:** 217–226.
20. Maruo, K., T. Akaike, T. Ono, and H. Maeda. 1998. Involvement of bradykinin generation in intravascular dissemination of *Vibrio vulnificus* and prevention of invasion by a bradykinin antagonist. *Infect. Immun.* **66:** 866–869.
21. McCarter, L. L. 1998. OpaR, a homolog of *Vibrio harveyi* LuxR, controls opacity of *Vibrio parahaemolyticus*. *J. Bacteriol.* **180:** 3166–3173.
22. McDougald, D., S. A. Rice, and S. Kjelleberg. 2000. The marine pathogen *Vibrio vulnificus* encodes a putative homologue of the *Vibrio harveyi* regulatory gene, *luxR*: A genetic and phylogenetic comparison. *Gene* **248:** 213–221.
23. McDougald, D., S. A. Rice, and S. Kjelleberg. 2001. SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus*. *J. Bacteriol.* **183:** 758–762.
24. Muench, K. H. 1989. Hemochromatosis and infection: Alcohol and iron, oysters and sepsis. *Am. J. Med.* **87:** 40N–43N.
25. Nealson, K. H. and J. W. Hastings. 1979. Bacterial bioluminescence: Its control and ecological significance. *Microbiol. Rev.* **43:** 496–518.
26. Oliver, J. D. 1989. *Vibrio vulnificus*, pp. 569–600. *In* Doyle, M. (ed.), *Foodborne Bacterial Pathogens*. Marcel-Dekker, New York, U.S.A.
27. Paranjpye, R. N., J. C. Lara, J. C. Pepe, C. M. Pepe, and M. S. Strom. 1998. The type IV leader peptidase/N-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to HEp-2 cells and virulence in iron-overloaded mice. *Infect. Immun.* **66:** 5659–5668.
28. Park, J. W., S. N. Ma, E. S. Song, C. H. Song, M. R. Chae, B. H. Park, H. W. Rho, S. D. Park, and H. R. Kim. 1996. Pulmonary damage by *Vibrio vulnificus* cytolysin. *Infect. Immun.* **64:** 2873–2876.
29. Park, K. J., S. H. H. Kim, M. G. Kim, D. H. Chung, S. D. Ha, K. S. Kim, D. J. Jahng, and K. H. Lee. 2004. Functional complement of *Escherichia coli* by the *rpoS* gene of the foodborne pathogenic *Vibrio vulnificus*. *J. Microbiol. Biotechnol.* **14:** 1063–1066.
30. Poper, K. R., S. Beck von Bodman, and S. K. Farrand. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362:** 448–450.
31. Reed, L. J. and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27:** 493–497.
32. Rhee, J. E., H. M. Ju, U. Y. Park, B. C. Park, and S. H. Choi. 2004. Identification of the *Vibrio vulnificus cadC* and evaluation of its role in acid tolerance. *J. Microbiol. Biotechnol.* **14:** 1093–1098.
33. Rhee, J. E., J. H. Lee, H. S. Jeong, U. Y. Park, D. H. Lee, G. J. Woo, S. I. Moyoshi, and S. H. Choi. 2003. Evidence that temporally alternative expression of the *Vibrio vulnificus* elastase prevents proteolytic inactivation of hemolysin. *J. Microbiol. Biotechnol.* **13:** 1021–1026.
34. Sambrook, J., E. F. Fritsch, and T. A. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd Ed. Cold Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.
35. Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler. 2001. The LuxS family of bacterial autoinducers: Biosynthesis of a novel quorum-sensing signal molecule. *Mol. Microbiol.* **41:** 463–476.
36. Shao, C. P. and L. I. Hor. 2000. Metalloprotease is not essential for *Vibrio vulnificus* virulence in mice. *Infect. Immun.* **68:** 3569–3573.
37. Shao, C. P. and L. I. Hor. 2001. Regulation of metalloprotease gene expression in *Vibrio vulnificus* by a *Vibrio harveyi* LuxR homologue. *J. Bacteriol.* **183:** 1369–1375.
38. Simpson, L. M. and J. D. Oliver. 1987. Ability of *Vibrio vulnificus* to obtain iron from transferring and other iron-binding proteins. *Curr. Microbiol.* **15:** 155–157.
39. Skorupski, K. and R. K. Taylor. 1996. Positive selection vectors for allelic exchange. *Gene* **169:** 47–52.
40. Stintzi, A., K. Evans, J. M. Meyer, and K. Poole. 1998. Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: *lasR/lasI* mutants exhibit reduced pyoverdine biosynthesis. *FEMS Microbiol. Lett.* **166:** 341–345.
41. Swift, S., A. V. Karlyshev, L. Fish, E. L. Durant, M. K. Winson, S. R. Chhabra, P. Williams, S. Macintyre, and G. S. Stewart. 1997. Quorum sensing in *Aeromonas hydrophilia* and *Aeromonas salmonicida*: Identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. *J. Bacteriol.* **179:** 5271–5281.
42. Testa, J., L. W. Daniel, and A. S. Kreger. 1984. Extracellular phospholipase A2 and lysophospholipase produced by *Vibrio vulnificus*. *Infect. Immun.* **59:** 192–197.
43. Wright, A. C., L. M. Simpson, and J. D. Oliver. 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. *Infect. Immun.* **34:** 503–507.
44. Wright, A. C., J. L. Powell, J. B. Kaper, and J. G. Morris. 2001. Identification of a group 1-like capsular polysaccharide operon for *Vibrio vulnificus*. *Infect. Immun.* **69:** 6893–6901.
45. Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate. 1993. *Agrobacterium* conjugation and gene regulation by N-acyl-L-homoserine lactones. *Nature* **362:** 446–448.
46. Zhu, J., M. B. Miller, R. E. Vance, M. Dziejman, B. L. Bassler, and J. J. Mekalanos. 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **99:** 3129–3134.