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Identification and Functional Analysis of *Vibrio vulnificus* SmcR, a Novel Global Regulator

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Abstract Recently, quorum sensing has been implicated as an important global regulator controlling the production of numerous virulence factors such as capsular polysaccharides in bacterial pathogens. The nucleotide and deduced amino acid sequences of *smcR*, a homolog of *V. harveyi luxR* identified from *V. vulnificus* ATCC29307, were analyzed. The amino acid sequence of SmcR from *V. vulnificus* was 72 to 92% similar to those of LuxR homologs from *Vibrio* spp. Functions of SmcR were assessed by the construction of an isogenic mutant, whose *smcR* gene was inactivated by allelic exchanges, and by evaluating its phenotype changes *in vitro* and in mice. The disruption of *smcR* resulted in a significant alteration in biofilm formation, in type of colony morphology, and in motility. When compared with the wild-type, the *smcR* mutant exhibited reduced survival under adverse conditions, such as acidic pH and hyperosmotic stress. The *smcR* mutant exhibited decreased cytotoxic activity toward INT 407 cells *in vitro*. Furthermore, the intraperitoneal LD_{50} of the *smcR* mutant was approximately 10^2 times higher than that of parental wild-type. Therefore, it appears that SmcR is a novel global regulator, controlling numerous genes contributing to the pathogenesis as well as survival of *V. vulnificus*.

Key words: *Vibrio vulnificus*, *smcR*, novel global regulator

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of foodborne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with underlying predisposing conditions such as liver damage, excess levels of iron, and immunocompromised conditions [16, 34]. Wound infections result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first signs of illness [16, 34].

Microbial pathogenicity is multifactorial and a complex phenomenon that involves the products of many genes. It has been generally accepted that virulence factors include all those factors contributing to survival and multiplication on or within a host as well as to disease [20]. Most of these virulence factors act cooperatively to obtain maximum effectiveness in the pathogenesis, and their expression is coordinately controlled by a common global regulatory system in response to environmental signals [22]. This coordinated regulation by global regulators would facilitate cooperation of the virulence factors, and would be crucial for the overall success of the infectious microorganisms during pathogenesis [7]. Several potential virulence factors, including an endotoxin, polysaccharide capsule, iron sequestering systems, cytolytic hemolysin, elastase, phospholipase A2, and other exotoxins, have been identified in *V. vulnificus* [16, 34].

It has long been recognized that many bacteria monitor their cell-population densities through the exchange of diffusible signal molecules (called autoinducers) that accumulate extracellularly [5, 21]. This type of communication, termed quorum sensing, effectively enables the bacteria to modulate their behavior and coordinately alter expression of a set of genes in a cell-density-dependent manner. The cell-density-dependent regulation of bioluminescence in *V. harveyi* is frequently used as a model for quorum sensing. *V. harveyi* LuxR is the transcriptional regulator of the luminescence operon. Recently, increasing numbers of LuxR

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homologs have been identified in different *Vibrio* spp., such as *V. cholerae*, *V. parahaemolyticus*, *V. anguillarum*, and *V. vulnificus* [2, 9, 12, 13, 17, 19]. Although LuxR homologs from *Vibrio* spp. exhibit high levels of identity (72–92% in amino acid sequences) [18], the specific features of their functions clearly differ, such that the regulatory proteins act as a repressor with some promoters and as an activator with others. Null mutations of LuxR result in a broad range of pleiotropic phenotype changes, indicating that LuxR homologs are apparently novel global regulators whereby the transcription of a set of different genes is regulated.

Recently, quorum sensing has also been implicated as an important global regulator controlling the expression of numerous virulence factors in bacterial pathogens [35]. It has been postulated that the pathogens regulate expression of different sets of virulence genes by sensing their own population density higher than critical levels, which can be obtained only in specific locations within the host. *V. vulnificus* SmcR is a homolog of *V. harveyi* LuxR. Indeed, SmcR appears to be involved in the regulation of virulence gene expression [7, 33]. However, to date, only a few phenotypes that are modulated by SmcR have been reported [18]. Accordingly, we extend our efforts here to characterize the functions of SmcR by examining phenotypes modulated by SmcR. For this purpose, the nucleotide and deduced amino acid sequence of *smcR*, an open reading frame identified from *V. vulnificus* ATCC29307, were analyzed. In addition, a *V. vulnificus* null mutant, in which the *smcR* gene was inactivated, was constructed by allelic exchanges, and the possible roles of the SmcR protein in regulation of the virulence as well as in stress tolerance of *V. vulnificus* have been demonstrated.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless noted otherwise, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). For measurement of cell growth, cultures of *V. vulnificus* strains were grown at 30°C with aeration. All the media components were purchased from Difco (Detroit, MI, U.S.A.), and the chemicals from Sigma (St. Louis, MO, U.S.A.).

General Genetic Methods

Procedures for the isolation of plasmid DNA, genomic DNA, and transformation were carried out as described by Sambrookand Russell [30]. Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, MA, U.S.A.). DNA fragments were purified from agarose gels using the Geneclean II kit (Bio 101, Inc., Vista, CA, U.S.A.). Primary DNA cloning and manipulation were conducted in *E. coli* DH5α, and restriction mapping was used to confirm that transformants contained the appropriate plasmids. PCR amplification of DNA was performed using a GeneAmp PCR system 2400

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	Laboratory collection
HS03	ATCC29307, *smcR*::*nptI*	This study
E. coli		
DH5α	*supE44 ΔlacU169* (ϕ80 *lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10 λ*pir*	*Thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*, *oriT* of RP4, Km^r; conjugational donor	[23]
Plasmids		
pUC18	Cloning vector; Ap^r	Laboratory collection
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	[25]
pRK415	Broad-host-range vector, IncP *ori*, *oriT* of RK2; Tc^r	[11]
pCVD442	R6K γ *ori*, *sacB*, *oriT* of RP4; Ap^r	[4]
pHS103	pUC18 with *smcR*; Ap^r	This study
pHS1031	pHS103 with *nptI*; Ap^r, Km^r	This study
pHS1032	pCVD442 with *smcR*::*nptI*	This study
pHS105	pRK415 with *smcR*; Tc^r	This study

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

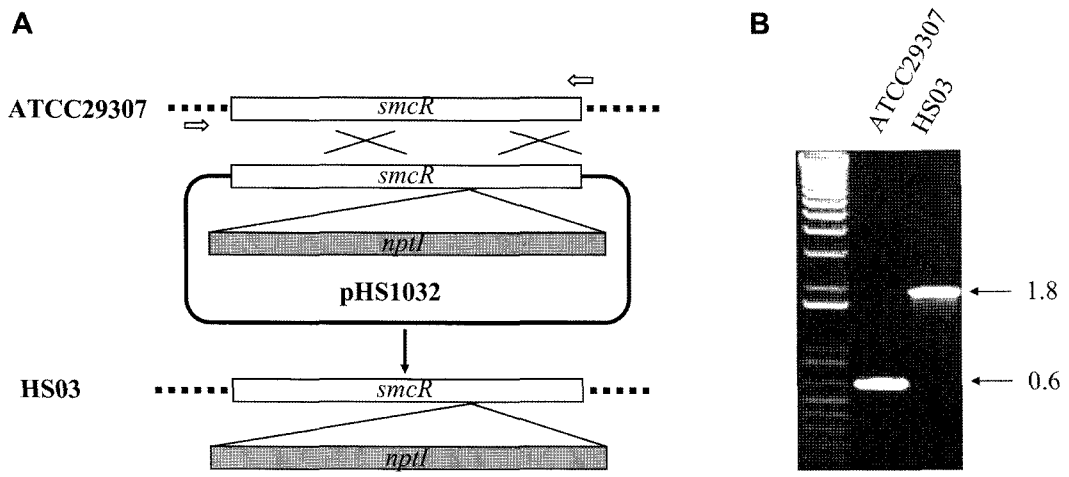

Fig. 1. Allelic exchange procedure and construction of *smcR::nptI* isogenic mutant.
A. Double homologous recombinations between strain ATCC29307 and plasmid pHS1032 led to interruption of the *smcR* gene and resulted in construction of the mutant HS03. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open box, the target *smcR* gene; shaded box, the *nptI* gene; open arrows, locations of the oligonucleotide primers used for confirmation of the *nptI* insert; large X's represent genetic crossing over. Abbreviations; *sacB*, levansucrase gene; *bla*, β-lactamase gene. **B.** PCR analysis of ATCC29307 and isogenic mutant HS03 generated by allelic exchange. Molecular size markers (1-kb plus DNA ladder, GIBCO-BRL) and PCR products are indicated.

(Perkin-Elmer, Norwalk, CT, U.S.A.) following standard protocols.

Generation of *smcR::nptI* Mutant

A 1.2-kb DNA fragment carrying the *smcR* ORF was cloned in pHS103 (Table 1). To inactivate the *smcR in vitro*, 1.2-kb *nptI* DNA conferring resistance to kanamycin [25] was inserted into a unique ClaI site present within the *smcR* ORF. The 2.4-kb *smcR::nptI* cartridge was then liberated from the resulting construct (pHS1031) and ligated with SmaI-digested pCVD442 [4] to form pHS1032 (Fig. 1A, Table 1). To generate the *smcR::nptI* mutant by homologous recombination, *E. coli* SM10 λ *pir*, *tra* (containing pHS1032) [22] was used as a conjugal donor to *V. vulnificus* ATCC29307. The conjugation and isolation of the transconjugants were conducted using previously described methods [26, 29].

Biofilm Formation Assay

Biofilm formation was assessed using a 96-well microtiter plate as described previously [24], with minor modifications. Briefly, an aliquot (7.5 μl) of *V. vulnificus* strains grown and adjusted to OD_{600} 1.0 in LBS was inoculated into 150 μl of autoinducer bioassay (AB, 6) broth placed in a 96-well polystyrene microtiter plate and incubated at 30°C for 48 h without shaking. Bacterial cultures were poured out and washed three times with PBS (pH 7.4), and then stained with 170 μl of 1% crystal violet solution for 15 min at room temperature. The bands of adherent cells on the wall of the plate were washed twice with distilled water, air-dried, and photographed. Quantitative biofilm measurement was assessed based on intensities of each band, using a UMAX digital imaging system (UTA-1100, UMAX Technologies, Inc. Fremont, CA, U.S.A.) and Kodak 1D Image Analysis software (Eastman Kodak Co., Rochester, NY, U.S.A.). Microscopic analysis for biofilm formation was performed by the same procedure as described above, except that *V. vulnificus* strains were inoculated onto glass coverslips placed at the bottom of the petridish. The bacterial biofilm on the coverslips was examined under a light microscope at a magnification of ×400.

Colony Morphotypes and Motility Test

For examining colony morphotypes, *V. vulnificus* strains were grown on an HI (Bacto Heart Infusion, Difco) plate, which provides an excellent contrast for the opaque and translucent colony types. Colony morphotypes were photographed by using a UMAX digital imaging system. For the motility test, *V. vulnificus* strains were grown overnight, and subsequently, equal amounts of the strains were stabbed into LBS semisolid media solidified with 0.3% agar (Bacto Agar, Difco) [10]. The plates were incubated at 30°C, and migration through the agar was monitored over a period of 24 h and photographed by the same procedure as described above.

Cytotoxicity Assay

Two different cytotoxicity assays were performed using INT-407 (ATCC CCL-6) human intestinal epithelial cells. The *V. vulnificus* strains were grown in an LBS broth, harvested by centrifugation, and suspended in a cell culture medium, MEM (minimum essential medium) containing 1% (v/v) fetal bovine serum (GIBCO-BRL,

Gaithersburg, MD, U.S.A.), to appropriate concentrations. The preparation of the INT-407 cells and infection with the bacterial cultures were performed in a 96-well tissue culture plate (Nunc, Roskilde, Denmark) as described previously [14, 26]. The cytotoxicity was then determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany), and expressed using the total LDH activity of the cells completely lysed by 1% Triton-X 100 as 100%. Morphologic studies were also carried out using INT-407 cells, which were seeded onto glass coverslips placed at the bottom of the tissue culture plate and infected with the *V. vulnificus* strains at an MOI of 90 for 30 min. The cells were fixed in methanol, stained with 0.4% Giemsa, and examined under a light microscope [26].

Mouse Model of Infection

The 50% lethal doses (LD_{50}s) of wild-type and the *smcR* mutant were compared using ICR mice (Specific Pathogen-Free; Daehan Animal Co., Taejon, Korea), as described elsewhere [8, 15]. For the determination of LD_{50}, bacteria grown in LBS broth overnight at 30°C were harvested and suspended in PBS to appropriate concentrations, ranging from 10^1 to 10^6 CFU in 10-fold increments.

A group of (n=6) 7-weeks-old normal female mice were injected intraperitoneally with 0.1 ml of serial dilutions of bacterial suspensions. The infected mice were observed for 24 h, and the LD_{50}s were calculated by the method of Reed and Muench [27]. Mice were injected intraperitoneally with 250 μg of iron dextran per g of body weight immediately before injection with bacterial cells.

Stress Tolerance Assay

Stress tolerance was assessed by measuring survivals in a challenged condition. *V. vulnificus* strains were grown to exponential phase (OD_{600} 0.8) in LBS and used as inocula as indicated. For acid tolerance, a 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 LBS as previously described [28]. The percentage of survivors was calculated relative to the CFU/ml as determined immediately after inoculation as 100%.

For comparison of tolerance to high osmolarity, the survivals of *V. vulnificus* strains were measured in LB plate supplemented with 0.6 M NaCl as a source of osmotic stress. Inocula then removed were used to spread in triplicate on LBS plate and LB plate with 0.6 M NaCl at the same time. Following incubation at 30°C overnight, the percentage of survivors on LB plate with 0.6 M NaCl was calculated by using the CFU/ml as appeared on LBS as 100%.

Data Analysis

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 Student's unpaired *t* test (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Significance was accepted at $p<0.05$.

RESULTS

Cloning and Sequence Analysis of *V. vulnificus smcR*

To assess the functions of *V. vulnificus* SmcR, the *smcR* gene was cloned from strain ATCC29307. A DNA fragment encompassing the *smcR* coding region was amplified from genomic DNA of *V. vulnificus* by a PCR using a pair of oligonucleotide primers. The primers (SmcR001, 5'-CCACGTAGCAAGCCGACTAG-3', sense primer, and SmcR002, 5'-CGGCCCCACGTTTTGCACTG-3', antisense primer) were designed using the genome sequence of *V. vulnificus* YJ016 (GenBank Accession No. NC_005139, www.ncbi.nlm.nih.gov) and synthesized (Takara, Seoul, Korea). A 1.2-kb-DNA fragment containing the *smcR* structural gene and upstream regulatory region was amplified by PCR from genomic DNA of *V. vulnificus* ATCC29307. The PCR product was purified from gel, blunt-ended, and then ligated into pUC18 linearized with SmaI to result in pHS103.

The nucleotide sequence of the 1.2-kb DNA fragment in pHS103 was then determined by primer walking (Korea Basic Science Institute, Gwang-Ju, Korea). The nucleotide sequence of *smcR* from *V. vulnificus* ATCC29307 was deposited in the GenBank database under Accession No. AAK29753. The nucleotide sequence revealed a coding region consisting of 618 nucleotides. The amino acid sequence deduced from the *smcR* coding sequence revealed a protein, SmcR composed of 205 amino acids with a theoretical molecular mass of 23,717 Da and pI of 5.81. The amino acid composition and molecular weight of this SmcR are quite similar to those of the LuxR homologs from *Vibrio* spp. The amino acid sequence of the *V. vulnificus* SmcR was 72% to 92% identical to those of the LuxR homologs from *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, and *V. anguillarum*, and their identity appeared evenly throughout the whole proteins (data not shown, http//www.ebi.ac.uk/clustalw).

Construction and Confirmation of *V. vulnificus smcR* Mutant

A double crossover, in which the wild-type *smcR* gene was replaced with the *smcR*::*nptI* allele, was confirmed by a PCR using a pair of primers, SmcR003 (5'-ATGGACTCAATCGCAAAGA-3') and SmcR004 (5'-TAGGCGTGCTCGC GTTTA-3') (Fig. 1B). The PCR

analysis of the genomic DNA from ATCC29307 with the primers produced a 0.6-kb fragment (Fig. 1B), whereas the genomic DNA from the *smcR*::*nptI* mutant resulted in an amplified DNA fragment approximately 1.8-kb in length. The 1.8-kb fragment was in agreement with the projected size of the DNA fragment containing the wild-type *smcR* (0.6-kb) and the *nptI* (1.2-kb) genes. The *V. vulnificus smcR* mutant chosen for further analysis was named HS03, as shown in Fig. 1B.

Effect of Mutation in *smcR* on Biofilm Formation

To determine if SmcR is required for biofilm formation, the *smcR* mutant HS03 was tested for its ability to form a biofilm on a polystyrene surface compared with the wild-type. The parental wild-type strain ATCC29307 was able to form a biofilm on polystyrene. On the other hand, the ability of HS03 to form a biofilm was significantly reduced on polystyrene surfaces (Fig. 2A). Based on the intensities of the bands, the residual level of the biofilm corresponded to approximately a half of that in wild-type (Fig. 2A). To further characterize the effects of SmcR on biofilm formation, biofilms developed on glass by suspending coverslips into cultures of *V. vulnificus* were analyzed microscopically (Fig. 2B). Wild-type was able to develop biofilm on the glass surface. The density of the cells aggregated in the biofilm was high and the biofilm structure was extensively spread throughout the coverslips. However, the *smcR* mutant appeared to form less biofilm with a decreased number of cells on the glass surface. The structure of the biofilm with mutant cells was not as extensive as the wild-type biofilm, and instead, was not equally dispersed on the surface of the coverslips. Taken together, these results suggest that the *V. vulnificus smcR* is involved in biofilm formation.

To rule out the possibility that the decreased biofilm formation resulted from polar effects of the *smcR* insertional mutation on downstream genes, we examined if reintroduction of pHS105 (Table 1) carrying a recombinant *smcR* could complement the decreased biofilm formation of HS03 cells. For this purpose, pHS105 was constructed by subcloning the *smcR* amplified by PCR using primers SmcR005 (5'-GAATCTAGAAAGGAAACAACCTATG-GACTC-3') and SmcR006 (5'-GAAGAATTCCCTT GATATGAGGTTACTGG-3') and then digested with XbaI and EcoRI into the broad-host-range vector pRK415 [11] linearized with the same enzymes. The resulting plasmid was mobilized into *V. vulnificus* by conjugation. The biofilm formation of the HS03 (pHS105) was restored to a level equivalent to, and even higher than, the wild-type level of ATCC29307 (Fig. 2A), and the overall profile of biofilm formation of the complemented strain was comparable to that of ATCC29307 (Fig. 2B). Therefore, the decreased biofilm formation of HS03 resulted from inactivation of functional *smcR* rather than any polar effects on any genes downstream of *smcR*.

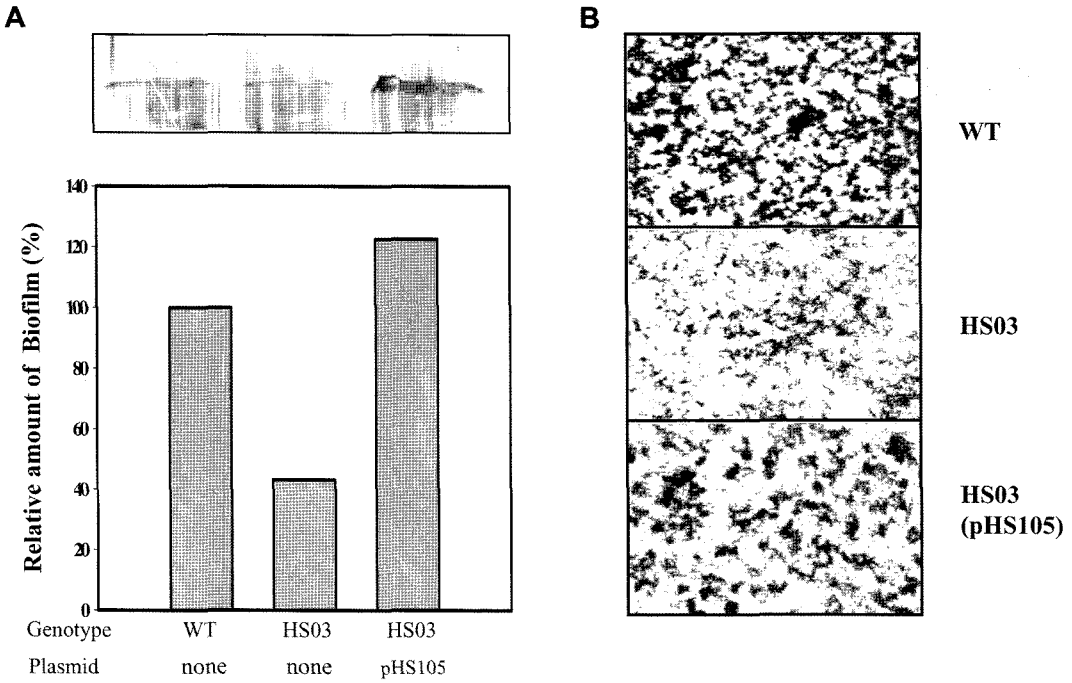

Fig. 2. Biofilm formation ability of *V. vulnificus* strains.

A. Biofilm on the wall of the polystyrene microplate well was stained with crystal violet. Relative amounts of the biofilm of each band were presented using the amount of the band of wild-type as 100%. **B.** Micrograph showing the biofilm formation on glass coverslips. Bacteria on coverslips were stained with crystal violet and visualized with a light microscope (×400). For both panels, WT, ATCC29307; HS03, *smcR* mutant; HS03 (pHS105), complemented strain.

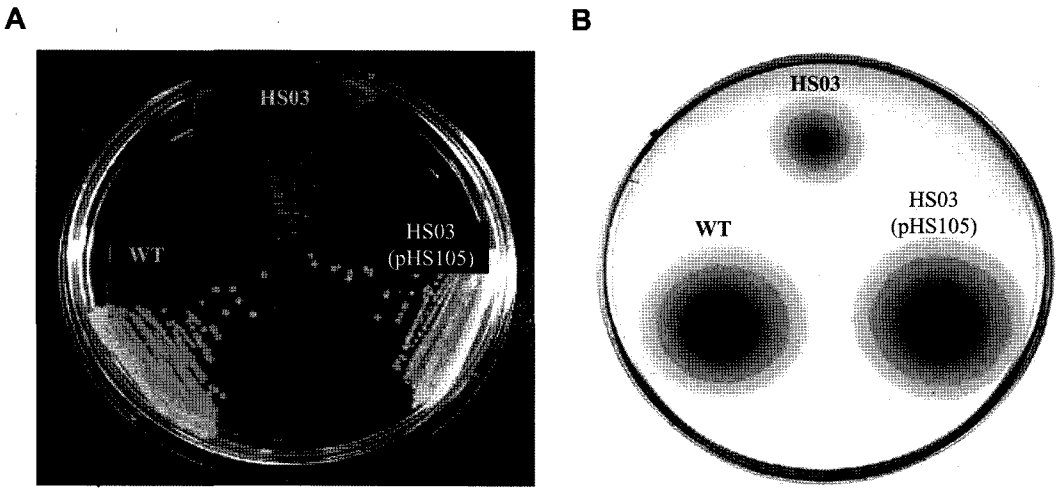

Fig. 3. Colony morphotypes and motility of *V. vulnificus* strains.
A. Colonies were grown on HI agar plates for one day and photographed by using a UMAX digital imaging system (UTA-1100, UMAX Technologies, Inc.). **B.** Wild-type strain (ATCC29307) displays motility in LBS containing 0.3% agar, which is reduced by mutation in *smcR* (HS03). For both panels, WT, ATCC29307; HS03, *smcR* mutant; HS03 (pHS105), complemented strain.

Effect of Mutation in *smcR* on Colony Morphotypes and Motility

It has been demonstrated that *V. vulnificus* expresses numerous gene products that are postulated to contribute to the biofilm formation. Among those factors is an exopolysaccharide (EPS, capsular polysaccharide). To examine if the differences in biofilm formation were associated with the alteration in EPS production, we examined the colony morphology of the wild-type and the *smcR* mutant (Fig. 3A). After growth on HI for one day, the wild-type showed opaque colonies. In contrast, colonies of the *smcR* mutant were significantly more translucent, indicating appreciable amounts of EPS are deficient on the surface of cells. Taken together, these results indicated that the EPS deficiency in *smcR* mutant would be a factor responsible for its reduced ability to form biofilm.

It has been demonstrated that motility is one of the mechanisms by which the bacteria can get the appropriate niche inside the host after infection. Several studies have also demonstrated that motility of pathogenic bacteria facilitates biofilm formation and colonization on host epithelial cells. To determine if the *smcR* in *V. vulnificus* is required for motility, the *smcR* mutant HS03 was tested for its ability to migrate on a semisolid plate surface compared with the wild-type. As shown in Fig. 3B, the growth away of *smcR* mutant from the inoculation point decreased compared with the wild-type, and the swimming diameter of the mutant was consistently reduced about 50% of that of the wild-type. This result indicated that SmcR is necessary for optimum motility of *V. vulnificus*.

SmcR is Required for Cytotoxicity to Epithelial Cells *In Vitro*

Since EPS production and biofilm formation and motility of pathogenic bacteria have been postulated to be important for virulence, it was a reasonable speculation that SmcR might also have a role in the virulence of *V. vulnificus*. In order to examine the effect of the *smcR* mutation on the virulence of *V. vulnificus*, LDH activities were determined from monolayers of INT-407 cells that were infected with 20 μl of suspension of ATCC29307 and HS03 strains at a different multiplicity of infection (MOI) and incubated for 3 h (Fig. 4A). The *smcR* mutant HS03 exhibited much less LDH activity provided that the MOI was not higher than 50. The level of LDH activity from INT-407 cells infected with HS03 at a MOI of 5 was almost 2-fold less than that from the cells infected with wild-type. In a similar way, INT-407 cells were infected at an MOI of 5, and LDH activity from the cells was compared at different incubation times as indicated (Fig. 4A). The cells infected with HS03 released a lower level of LDH activity than that of the cells infected with wild-type while the cells were incubated with bacterial suspension as long as 3 h. The lower LDH activities were restored to the level obtained from the cells infected with wild-type, when the cells were incubated with HS03 (pHS015).

To further investigate the cytotoxicity of the *smcR* mutant HS03 and its parental wild-type, morphological studies were also carried out using the INT-407 cells infected with *V. vulnificus* strains (Fig. 4B). Microscopic observation of INT-407 cells infected with ATCC29307 and HS03 at an MOI of 90 for 30 min was carried out as previously described [14]. The stained cells were assessed for size, regularity of the cell margin, and morphological characteristics of the nuclei. As shown in Fig. 4B, many Giemsa-stained INT-407 cells had marked cellular damage after infection with wild-type. Cytoplasmic loss and nuclear material condensation, typical phenotypes of cell death, were observed in the intestinal cells infected with wild-

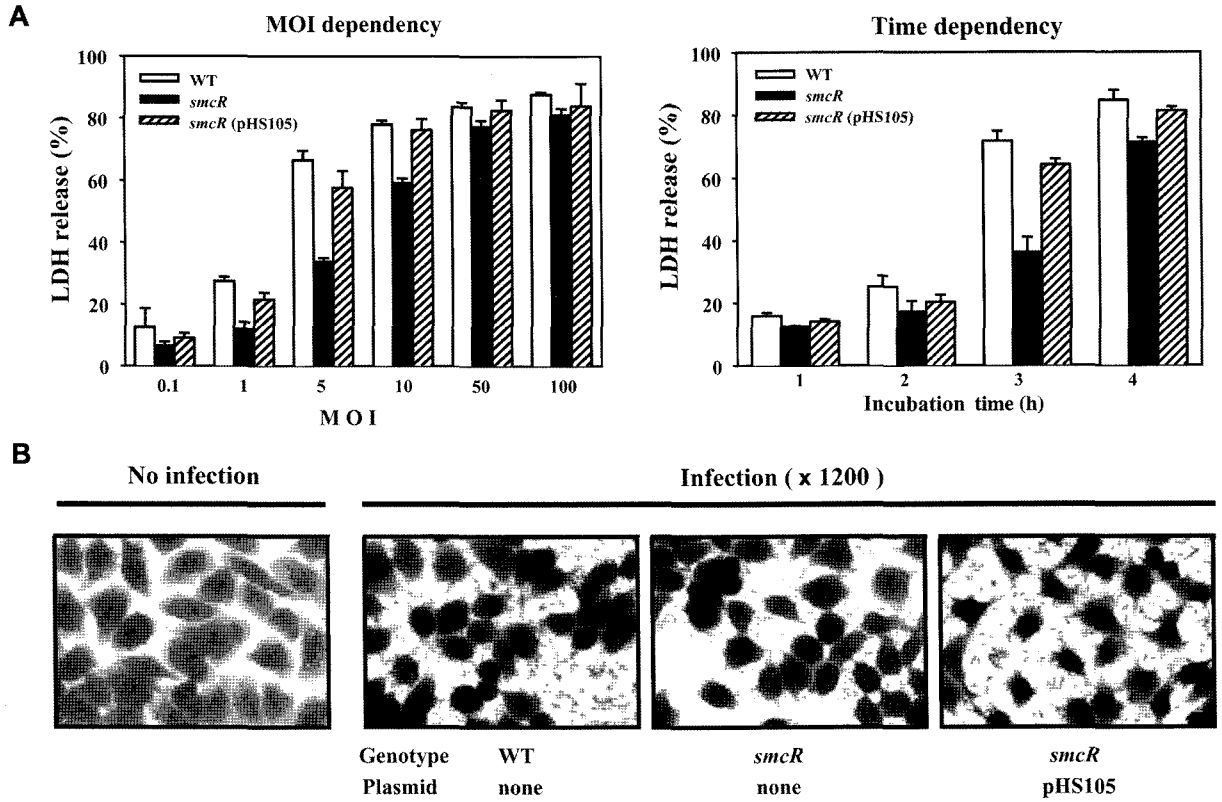

Fig. 4. Effect of the *smcR* mutation on the virulence of *V. vulnificus* to INT-407 cells.
A. INT-407 cells were infected with wild-type, *smcR* mutant, or complemented strain of *V. vulnificus* at various MOIs for 3 h (left), or at an MOI of 5 for various incubation times (right). Thereafter, the cell cytotoxicity was determined by an LDH release assay. The data represent the mean±SEM from three independent experiments. **B.** Microscopic observation of INT-407 infected with the *V. vulnificus* strains at an MOI of 90 for 30 min. From the left, uninfected (control) and infected with wild-type (WT), HS03 (*smcR*), or the complemented strain.

type and HS03 (pHS105). A lot of *V. vulnificus* cells were observed in the disrupted cytoplasmic region of the cells infected with wild-type and HS03 (pHS105). In contrast, less dead cells were observed after incubation with HS03. The cells infected with HS03 showed a less damaged surface and less cytoplasmic loss. Taken together, these results suggest that SmcR is important for *V. vulnificus* to infect and injure host cells.

Virulence in Mice is Dependent on *smcR*

The role of the *V. vulnificus smcR* gene in virulence was further examined using a mouse model. The LD_{50}s in iron-overloaded mice after intraperitoneal infections of *V. vulnificus* strains are shown in Table 2. The LD_{50} of HS03 was greater than 10^3 CFU, compared with an LD_{50} lower than 10^2 CFU for the wild-type. Therefore, in the mouse model of intraperitoneal infection, in which the *smcR* mutant showed approximately 2-log increase in LD_{50} over the wild-type, the *smcR* mutant appeared to be virtually less virulent than its parental wild-type. Taking these results together, it is reasonable to conclude that the *smcR* is essential for virulence of *V. vulnificus* in mice as in well as *in vitro* cell culture.

Table 2. Effects of the *smcR* mutation on the lethality of *V. vulnificus* to mice[a].

Strain	Intraperitoneal LD_{50} (CFU)
ATCC29307 (n=6)	1.4×10^1
HS03 (n=6)	1.6×10^3

[a]: n, number of iron-treated mice for each inoculation group, ranging from 10^1 to 10^6 CFU in 10-fold increments.

Effect of *smcR* 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 *smcR* mutant (HS03) when challenged with acidic pH (pH 4.4; Fig. 5A). The parent strain decreased ca.1.0 $\log_{10}$ CFU/ml (90%), while the mutant strain decreased ca. 3.0 $\log_{10}$ CFU/ml (99.9%) 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 HS03 when challenged with hyperosmolarity (5% NaCl) (Fig. 5B). This indicated that the log-phase cell of the *smcR* mutant

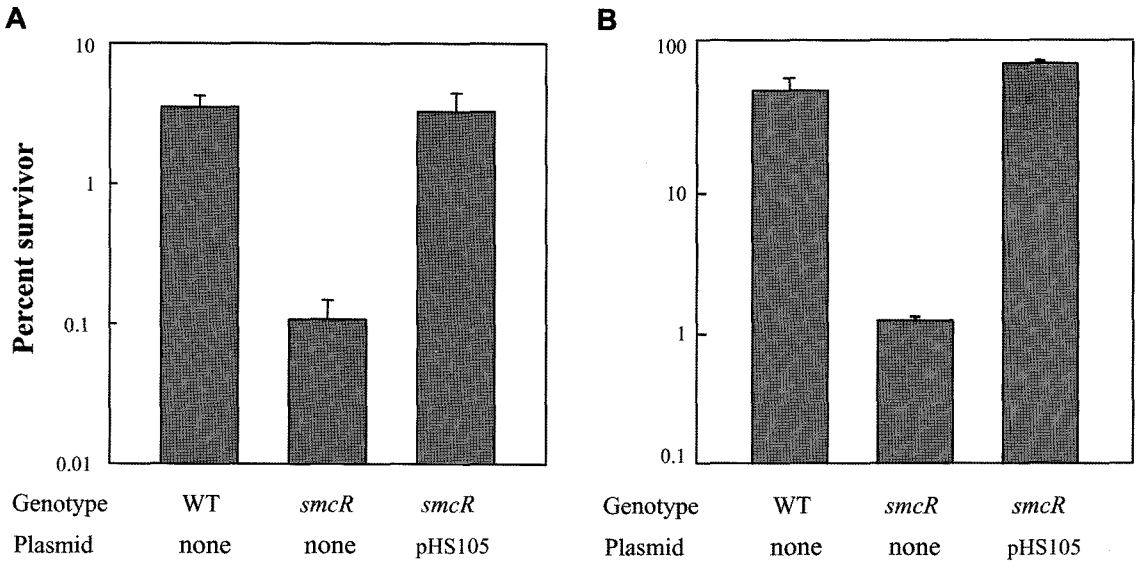

Fig. 5. Stress tolerance of *V. vulnificus* strains grown to log phase.
Survival of log-phase *V. vulnificus* parent strain (ATCC 29307), *smcR* mutant (HS03), and complemented strain. Strains were challenged with acidic pH (**A**) and hyperosmolarity (**B**) as described in the text. All results represent the mean from three independent trials. Error bars represent the standard errors.

was more sensitive to stresses than the wild-type, and that the SmcR plays an important role in survival of *V. vulnificus*.

Stationary-phase cells (A_{600}=2.0) of both ATCC29307 and HS03 exhibited a slower rate of decline and had a greater number of survivors after acid challenges than log-phase cells (data not shown). Additionally, compared with the results with cells of log phase, smaller differences in the survival of the parent strain and the *smcR* mutant were observed with stationary-phase cells. Consistent with this, the survival of stationary-phase cells of the parent strain, ATCC29307, and HS03 under hyperosmotic stress was not significantly different, indicating that the role of SmcR in survival under high osmotic stress is not crucial in cells of stationary phase (data not shown). Consequently, it was apparent that the contribution of the *smcR* gene for the stress tolerance of *V. vulnificus* is substantial, but dependent on growth phases.

DISCUSSION

Recent work has established that many species of bacteria regulate gene expression in response to signal molecules present in dense populations. This cell-density-dependent signaling phenomenon is collectively called quorum sensing. Quorum-sensing bacteria synthesize and release extracellular signaling molecules (AI, autoinducers) that accumulate in the environment as the bacterial population increases. When a threshold concentration of AI is achieved, a signal transduction cascade is triggered through cognate receptors, to modulate gene expression that may influence the virulence and a wide variety of phenotypes [3, 21]. Bioluminescence in *V. harveyi* is one of the best studied of the autoinducer-controlled gene expression systems, and regulated by two distinct AI signaling molecules. AI-1 is a well-characterized derivative of an *N*-acyl homoserine lactone (AHL), and AI-2 is a furanosyl borate synthesized by the product of *luxS* [1, 32]. *V. harveyi* LuxR is the transcriptional activator of the luminescence operon, and its synthesis is controlled by the density of the bacteria and the levels of both autoinducers [31].

This study has shown that disruption of *smcR* results in alterations in pleiotropic phenotypes, including biofilm formation, motility, and survival under various stresses. Although it is not yet clear whether SmcR acts in a cell-density manner or not, these results indicate that SmcR is apparently a global regulator in *V. vulnificus*. In addition to this, it is noted that the *smcR* mutant was less virulent. There have been substantial amounts of studies on the possible roles of the LuxR homologs in the virulence gene expression *in vitro* [35]. However, the major problem to be addressed is that those studies relied primarily on the analysis of expression of virulence factors *in vitro*. To date, no definitive analysis of the role of the LuxR homologs during an infectious process, rather than *in vitro* comparison of virulence gene expression, has been reported. In the present study, the function of SmcR during an infectious process has been examined by constructing an isogenic *smcR* mutant of *V. vulnificus* and comparing virulence of the mutant with that of parental wild-type. When compared with the wild-type, the *smcR* mutant is less toxic to intestinal epithelial cells *in vitro*, and also shows significantly diminished virulence in mice as measured by their abilities to cause death. These results led us to confirm that SmcR

is an important global regulator for genes associated with the survival and pathogenesis of *V. vulnificus*.

The nucleotide sequence of the *smcR* gene of *V. vulnificus* ATCC29307 was deposited into the GenBank under the accession number AAQ17507.

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