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Regulation of Cytotoxicity by Quorum-Sensing Signaling in *Vibrio vulnificus* Is Mediated by SmcR, a Repressor of *hlyU*▽†

Chung-Ping Shao,[1] Horng-Ren Lo,[2,3] Jen-Hsing Lin,[4] and Lien-I Hor[3,4]*

Department of Nursing, National Tainan Institute of Nursing, Tainan 700,[1] Department of Medical Technology, Fooyin University, Ta-Liao, Kaohsiung 831,[2] and Institute of Basic Medical Sciences,[3] Department of Microbiology and Immunology,[4] College of Medicine, National Cheng-Kung University, Tainan 701, Taiwan, Republic of China

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Cytotoxicity is an important virulence determinant in the pathogenesis of *Vibrio vulnificus*, and two cytotoxins, RTX (encoded by *rtxA1*) and cytolysin/hemolysin (encoded by *vvhA*), have been identified in this organism. We showed that the quorum-sensing regulator LuxO controlled the cytotoxicity of this organism: a Δ*luxO* mutant exhibited low cytotoxicity, whereas a constitutively activated *luxO* mutant, *luxO(D47E)*, remained highly cytotoxic. The cytotoxicity of the Δ*luxO* mutant was restored when *smcR*, a *Vibrio harveyi luxR* homologue repressed by *luxO*, was further deleted. SmcR then was shown to repress the expression of both *rtxA1* and *vvhA*. A DNA library of *V. vulnificus* was screened in *Escherichia coli* for clones that upregulated *vvhA* in the presence of SmcR, and *hlyU*, which has been shown to positively regulate *rtxA1* and *vvhA*, was identified. We demonstrated that SmcR repressed the expression of *hlyU* and bound to a region upstream of *hlyU* in *V. vulnificus*. The deletion of *hlyU* resulted in the loss of cytotoxicity and reduced cytolysin/hemolysin production in the Δ*smcR* mutant. The Δ*smcR* Δ*hlyU* mutant regained cytotoxicity and cytolysin/hemolysin activity when *hns*, which has been shown to repress the transcription of *rtxA1* and interfere with *hlyU*, was further removed. Collectively, our data suggest that SmcR mediates the regulation of cytotoxicity by quorum-sensing signaling in *V. vulnificus* by repressing *hlyU*, an activator of *rtxA1* and *vvhA*.

Vibrio vulnificus, a Gram-negative marine bacterium, is an opportunistic pathogen causing septicemia and wound infection in humans; it has a high mortality rate, particularly in those who suffer from chronic liver diseases or are immunocompromised. This organism produces two major cytotoxins, cytolysin/hemolysin (encoded by *vvhA*) and RTX (repeats in toxin; encoded by *rtxA1*), that are implicated in its virulence (19, 29). The cytolysin/hemolysin, which is an extracellular product, is lethal for mice at submicrogram levels. It lyses erythrocytes, increases vascular permeability (resulting in extensive extracellular edema in guinea pig skin), damages capillary endothelial cells, and causes mild inflammatory cell infiltration (7, 8). The RTX toxin, which forms pores on cell membranes only after the contact of the bacterium with the host cell (12, 13), is required for *V. vulnificus* virulence in mice by promoting bacterial colonization at the infection site and subsequent invasion into the bloodstream (12, 15).

Several regulators of *vvhA* and *rtxA1* have been identified (1, 19). HlyU, which was identified by *in vivo*-induced antigen technology and is essential for *V. vulnificus* virulence in mice (11, 19), is required for the expression of *vvhA* and *rtxA1* and binds directly to a region upstream of the operon where *rtxA1* is located (19, 20).

Quorum-sensing (QS) signaling, which is widely used by bacteria to communicate with each other, regulates the virulence genes in a variety of microorganisms, including *Vibrio* species (23, 30). In *Vibrio cholerae*, the signals transduced from at least three QS sensory circuits integrate into LuxO, a repressor of *hapR* (a *luxR* homologue) that negatively regulate the virulence regulon (30). The repression of *hapR* by LuxO is mediated by a set of QS-regulatory small RNAs, *qrr1-4*. In detail, the activated LuxO, together with RpoN, turns on the expression of *qrr1-4*, and these small RNAs in turn destabilize *hapR* mRNA in the presence of the *qrr*-binding protein Hfq (18).

The homologues of most genes of QS signaling in *V. cholerae*, including *luxS* (coding for the synthesis of autoinducer AI-2), *luxO*, *qrr1-4*, and *hapR*, are found in the whole-genome sequences of *V. vulnificus* strains YJ016 (2) and CMCP6. Kim et al. have reported that, in the absence of AI-2 (caused by a deletion in *luxS*), the production of protease was delayed and cytolysin/hemolysin production was increased. Further, these changes were complemented by AI-2 contained in the log-phase spent medium of the wild-type *V. vulnificus* (10). These findings suggest that the expression of both the protease and cytolysin/hemolysin is regulated by quorum-sensing signaling. However, whether QS is involved in the regulation of cytotoxicity mediated by cytolysin/hemolysin and RTX toxin remains unclear. Therefore, we designed experiments to determine if the cytotoxicity of *V. vulnificus* is regulated by QS and further explore the mechanism of regulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains and plasmids used are listed in Table 1, and the primers used are listed in Table S1 in the

* Corresponding author. Mailing address: Department of Microbiology and Immunology, College of Medicine, National Cheng-Kung University, Tainan 701, Taiwan, Republic of China. Phone: 886 6 2353535, ext. 5635. Fax: 886 6 2082705. E-mail: h061453@mail.ncku.edu.tw.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
V. vulnificus		
YJ016	Clinical isolate	Laboratory collection
CP156	YJ016 Δ*smcR*	27
YJ016Δ*smcR*Δ*vvp*	CP156 deleted of *vvp*	This study
YJ016Δ*rtxA1*	YJ016 deleted of *rtxA1*	21
YJ016Δ*luxO*	YJ016 deleted of *luxO*	This study
YJ016*luxO(D47E)*	YJ016 with *luxO* replaced by *luxO(D47E)*	This study
YJ016Δ*vvhA*	YJ016 deleted of *vvhA*	This study
YJ016Δ*luxO*Δ*smcR*	YJ016Δ*luxO* deleted of *smcR*	This study
YJ016Δ*luxO*Δ*smcR*::p*smcR*	YJ016Δ*luxO*Δ*smcR* with p*smcR* integrated into the vicinity of deletion in *smcR*; Ap^r	This study
YJ016Δ*luxO*Δ*smcR*Δ*vvhA*	YJ016Δ*luxO*Δ*smcR* deleted of *vvhA*	This study
YJ016Δ*luxO*Δ*smcR*Δ*vvhA*::p*smcR*	YJ016Δ*luxO*Δ*smcR*Δ*vvhA* with p*smcR* integrated into the vicinity of deletion in *smcR*; Ap^r	This study
YJ016Δ*luxO*Δ*smcR*Δ*rtxA1*	YJ016Δ*luxO*Δ*smcR* deleted of *rtxA1*	This study
YJ016Δ*luxO*Δ*smcR*Δ*rtxA1*::p*smcR*	YJ016Δ*luxO*Δ*smcR*Δ*rtxA1* with p*smcR* integrated into the vicinity of deletion in *smcR*; Ap^r	This study
YJ016Δ*luxO*Δ*smcR*Δ*vvhA*Δ*rtxA1*	YJ016Δ*luxO*Δ*smcR*Δ*vvhA* deleted of *rtxA1*	This study
YJ016Δ*luxO*Δ*smcR*Δ*vvhA*Δ*rtxA1*::p*smcR*	YJ016Δ*luxO*Δ*smcR*Δ*vvhA*Δ*rtxA1* with p*smcR* integrated into the vicinity of deletion in *smcR*; Ap	This study
YJ016*luxO(D47E)* Δ*smcR*	YJ016*luxO(D47E)* deleted of *smcR*	This study
YJ016Δ*vvhA*Δ*rtxA1*	YJ016Δ*rtxA1* deleted of *vvhA*	This study
YJ016Δ*lacZ*	YJ016 deleted of *lacZ*	This study
YJ016Δ*smcR*Δ*lacZ*	CP156 deleted of *lacZ*	This study
YJ016Δ*hlyU*	YJ016 deleted of *hlyU*	This study
YJ016 Δ*smcR*Δ*hlyU*	YJ016Δ*hlyU* deleted of *smcR*	This study
YJ016*luxO(D47E)* Δ*hlyU*	YJ016 *luxO(D47E)* deleted of *hlyU*	This study
YJ016Δ*luxO*Δ*smcR*Δ*hlyU*	YJ016Δ*luxO*Δ*smcR* deleted of *hlyU*	This study
YJ016Δ*hns*	YJ016 deleted of *hns*	This study
YJ016Δ*hns*::p*hns*	YJ016Δ*hns* with p*hns* integrated into the vicinity of deletion in *hns*; Ap^r	This study
YJ016Δ*hlyU*Δ*hns*	YJ016Δ*hlyU* deleted of *hns*	This study
YJ016Δ*hlyU*Δ*hns*::p*hns*	YJ016Δ*hlyU*Δ*hns* with p*hns* integrated into the vicinity of deletion in *hns*; Ap^r	This study
YJ016Δ*hlyU*Δ*smcR*Δ*hns*	YJ016Δ*hlyU*Δ*smcR* deleted of *hns*	This study
YJ016Δ*hlyU*Δ*smcR*Δ*hns*::p*hns*	YJ016Δ*hlyU*Δ*smcR*Δ*hns* with p*hns* integrated into the vicinity of deletion in *hns*; Ap^r	This study
E. coli		
DH5α	*supE44 lacU169*(ϕ80*lacZ*ΔM15) *hrdR17 recA1 endA1 gyrA96 thi-1 relA*	9
S17-1λ*pir*	*thi pro hsdR hsdM*$^+$ *recA*::RP4-2-Tc::Mu λ*pir* Km^r Nal^r	28
KV372	DH5α carrying pVH84	This study
Plasmids		
pJRD215	Shuttle vector; Km^r	3
pUC19	Cloning vector; Ap^r	Laboratory collection
pBR322	Cloning vector; Ap^r Tc^r	Laboratory collection
pGemT-easy	Cloning vector; Ap^r	Laboratory collection
pCVD442	Suicide vector; Ap^r	4
pΔ*luxO*	pCVD442 inserted with Δ*luxO*	This study
pΔ*rtxA1*	pCVD442 inserted with Δ*rtxA1*	21
pΔ*vvhA*	pCVD442 inserted with Δ*vvhA*	This study
pΔ*hlyU*	pCVD442 inserted with Δ*hlyU*	This study
pΔ*hns*	pCVD442 inserted with Δ*hns*	This study
pGemT-*hns*	pGemT-easy inserted with *hns*$^+$	This study
pOU1	pUC19 inserted with *luxO*$^+$	This study
p*luxO(D47E)*	pCVD442 inserted with *luxO(D47E)*	This study
p*smcR*	pCVD442 inserted with *smcR*$^+$	This study
p*hlyU*	pJRD215 inserted with *hlyU*$^+$	This study
p*hns*	pCVD442 inserted with *hns*$^+$	This study
pVR19	pCVD442 inserted with Δ*smcR*	27
pSI026	pCVD442 inserted with Δ*vvp*	26
pVP84	pJRD215 containing a promoterless *lacZ* gene of YJ016	Laboratory collection
pVH84	pVP84 inserted with a 675-bp, *vvhBA* promoter-containing DNA fragment in front of *lacZ*	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
pVU84	pVP84 containing 128 bp of *hlyU* promoter region in front of *lacZ*	This study
pVR11	pBR322 inserted with a 3.4-kb DNA fragment that contains *smcR*	27
pVR11.2	pBR322 inserted with a 1-kb *smcR*-containing DNA fragment cloned from pVR11	This study
pVR11.3	pBR322 inserted with Δ*smcR*	This study
pVR11.2-L24	pVR11.2 inserted with a 3.6-kb DNA fragment that contains ORFs VV0681, VV0682, and *hlyU*	This study
pVR11.2-L241	pVR11.2 inserted with a 3.6-kb fragment that contains ORF VV0681	This study
pVR11.2-L242	pVR11.2 inserted with a 2.4-kb fragment that contains ORF VV0682	This study
pVR11.2-L243	pVR11.2 inserted with a 2.38-kb fragment that contains ORFs VV0682 and *hlyU*	This study
pVR11.2-L244	pVR11.2 inserted with a 0.77-kb fragment that contains ORF VV *hlyU*	This study
pVR11.3-L244	pVR11.2-L244 Δ*smcR*	This study
pET30b	Protein expression vector; Km^r	Laboratory collection
pVR282	pET30b inserted with $smcR^+$ for producing C-terminally His_6-tagged SmcR	This study
pVR35	pJRD215 inserted with His_6-tagged $smcR^+$ cloned from pVR282	This study

[a] Abbreviations: Ap^r, Km^r, and Nal^r are resistance to ampicillin, kanamycin, and nalidixic acid, respectively.

supplemental material. The nucleotide sequences of primers were derived from the whole-genome sequence of *V. vulnificus* strain YJ016 (NCBI accession no. NC 005139-40). The bacteria were grown at 37°C with shaking at 240 rpm in Luria-Bertani (LB) medium, to which ampicillin (100 μg/ml), polymyxin B (50 U/ml), or kanamycin (50 μg/ml) was added as appropriate. Throughout this study, a 4-h culture at 37°C, starting from a 100-fold dilution of an overnight culture, was used to infect the cultured cells.

DNA preparation and manipulation. The plasmid and genomic DNAs were purified by a Wizard plasmid/genomic DNA purification kit (Promega, Madison, WI). Standard techniques were used to construct the recombinant plasmids (25). DNA restriction endonucleases and T4 DNA ligase were from New England BioLabs. PCR was performed as described previously (26) with a thermocycler (Mastercycler gradient; Eppendorf AG, Hamburg, Germany).

Isolation of *V. vulnificus* mutants and their complemented strains. *V. vulnificus* mutants with deletions in *luxO*, *rtxA1*, *vvhA*, *hlyU*, and *hns*, as well as the $luxO_{D47E}$ mutant, which contains an Asp-to-Glu mutation at the 47th residue that results in a constitutively active LuxO in *V. harveyi* (6), were isolated by *in vivo* allelic exchange (26). Briefly, for the isolation of deletion mutants, derivatives of suicide plasmid pCVD442, containing various DNA fragments with the desired deletions, first were constructed by the ligation of pCVD442 linearized by an appropriate restriction enzyme with the PCR-amplified upstream and downstream DNA fragments flanking the deletion. The corresponding primer pairs used to amplify the upstream (U) and downstream (D) DNA fragments were UVV1195F-UVV1195R and DVV1195F-DVV1196R for *luxO*, UVVA0965F1-UVVA0965R and DVVA0965F-DVVA0965R for *vvhA*, UVV0683F-UVV0683R and DVV0683F-DVV0683R for *hlyU*, and UVV1346F-UVV1346R and DVV1346F-DVV1346R for *hns* (see Table S1 in the supplemental material). The resultant plasmids were pΔ*luxO*, pΔ*vvhA*, pΔ*hlyU*, and pΔ*hns* (1,311, 1,032, 210, and 300 bp, respectively). In-frame deletions then were used to generate the specific gene knockout mutants. The Δ*rtxA1* mutants were isolated as described previously (21).

The *luxO(D47E)* mutant was isolated similarly, except that the mutation was introduced into pOU1, a pUC19 derivative containing the *luxO* gene amplified with primers vlo7 and vlo10, by a QuikChange site-directed mutagenesis kit (Stratagene, CA) and primers D47EF and D47ER (see Table S1). The pCVD442 derivative carrying the *luxO(D47E)* mutation (p$luxO_{D47E}$) was transferred to the Δ*luxO* mutant instead of the wild-type strain to facilitate the screening of mutants by PCR.

For the complementation of the Δ*smcR* mutant, a 2.1-kb NheI-NheI DNA fragment that contained the entire *smcR* gene was excised from pVR11 (pBR322 with *smcR* inserted) and was inserted into the XbaI site of pCVD442 to generate p*smcR*. This plasmid then was transferred to the Δ*smcR* mutants by conjugation, and the transconjugants with the whole plasmid integrated adjacent to the deletion in *smcR* by homologous recombination were selected. For the complementation of the Δ*hlyU* mutants, a 776-bp BglII-BamHI DNA fragment with the entire *hlyU* gene was cloned from pVR11.2-L24 (pBR322 derivative containing *hlyU*) into the BamHI site of pJRD215, a broad-host-range plasmid, and the recombinant plasmid then was transferred to the Δ*hlyU* mutants by conjugation. For the complementation of the Δ*hns* mutants, a 2,041-bp, *hns*-containing DNA fragment amplified by PCR with primers UVV1346F and DVV1346R was cloned into the SacI site of pCVD442. The resultant plasmid subsequently was transferred to the Δ*hns* mutants by conjugation, and the transconjugants with the whole plasmid integrated adjacent to the deletion in *hns* by homologous recombination were selected.

Cytotoxicity assay. Monolayers of HEp-2 cells, about 1.5×10^4 cells per well in a 96-well microplate, were infected with phosphate-buffered saline (PBS)-washed bacteria harvested from a 4-h culture at a multiplicity of infection (MOI) of 10 for 4 h. The infected monolayer then was assayed for cell lysis with a CytoTox 96 nonradioactive cytotoxicity kit (Promega) that measures the activity of lactate dehydrogenase (LDH) released from lysed cells. The cytotoxicity is expressed as 100 × [(LDH activity of infected monolayer − LDH activity of monolayer treated with medium)/(the LDH activity of monolayer treated with lysis buffer − LDH activity of monolayer treated with medium)].

Cytolysin/hemolysin assay. The amount of cytolysin/hemolysin produced in culture supernatant or conditioned medium of infected cells was assayed by the hemolysis of mouse red blood cells (RBCs). The sample was mixed with an equal volume of mouse RBC suspension (0.7 to 0.9% in PBS), and the mixture was incubated at 37°C for 30 min. The unlysed RBCs and cell debris were pelleted by centrifugation, and the optical density at 545 nm (OD_{545}) of the supernatant, which represents the level of hemolysis, was measured. The cytolysin/hemolysin level is expressed as 100 × (OD_{545} of RBC suspension treated with sample/OD_{545} of RBC suspension treated with 1% Triton X-100).

RNA isolation and cDNA synthesis. Total RNA was extracted from *V. vulnificus* cells by TRI reagent (Sigma-Aldrich), and the concentration of RNA was determined with a BioPhotometer (Eppendorf). DNA contaminants were removed by DNase (RQ1 RNase-free DNase; Promega) before RNA precipitation by ethanol. The cDNA of total RNA (2 μg per reaction mixture) was synthesized with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

Real-time PCR. Real-time PCR was performed with Mastercycler ep realplex (Eppendorf). The standard 1× real-time PCR mixture contained 12.5 μl 2× Power SYBR green PCR master mix (Applied Biosystems), 50 ng cDNA, and 200 nM (each) forward and reverse primers. The 23S rRNA gene was used as an internal control, and the experiment was performed in triplicate. The reaction consisted of an initial denaturing at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s and annealing at 60°C for 60 s. DNA polymerization was conducted in a range of temperatures from 60 to 95°C within 20 min to obtain the melting curve for determining the PCR amplification specificity. The threshold cycle (C_T) value and relative change in expression level were determined by automated threshold analysis with Realplex software, v. 1.5 (Eppendorf).

Construction of the reporter strains and β-galactosidase assay. The *Escherichia coli* DH5α reporter strains, which expressed *lacZ* under the control of various *V. vulnificus* promoters, were constructed for studying the regulation of these promoters. To construct the P*vvhBA-lacZ* fusion, a 675-bp DNA fragment that contained the promoter of the *vvhBA* operon and an NdeI restriction site with a start codon, ATG, was amplified by PCR with primers pvvhF and pvvhR. The PCR product was cloned into pVP84, which contained a promoterless *lacZ*

gene from *V. vulnificus*, at the NdeI site to create pVH84. For the construction of the P*hlyU-lacZ* fusion, a 299-bp DNA fragment that contained part of VV0682, the entire intergenic region upstream of *hlyU*, and an NdeI site with a start codon, ATG, was amplified by PCR with primers phlyUF and phlyUR. The PCR product then was cloned into pVP84 at the NdeI site to create pVU84. For β-galactosidase assay of *V. vulnificus*, pVU84 was transferred from *E. coli* S17-1λ*pir* to the various *V. vulnificus* strains by conjugation.

To assay the activity of the promoter fused with *lacZ* in a reporter strain, an overnight bacterial culture starting with a single colony picked from a 2-day plate culture was 100-fold diluted in fresh LB medium and incubated at 37°C with shaking. An aliquot of the culture then was collected at intervals, and the β-galactosidase activity in the culture supernatant was determined as described previously (22).

Library construction, screening, and subcloning. The genomic DNA was extracted from *V. vulnificus* YJ016 and partially digested with Sau3AI, and the DNA fragments ranging from 3 to 6 kb were recovered and ligated to the BamHI-linearized pVR11.2, a pBR322 derivative containing the intact *smcR* gene. The resultant plasmids were introduced into *E. coli* strain KV372, which carried pVH84 that contained the P*vvhBA-lacZ* fusion, by transformation to generate a DNA library for screening the gene(s) that could mediate the regulation of *vvhA*. The blue colonies, which indicated enhanced *vvhBA* promoter activity by the gene cloned into pVR11.2 on 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)-containing plates, were selected for further characterization.

Production and purification of SmcR with a His_6 tag at C terminus. The *smcR* gene with its promoter was amplified by PCR with primers VR22 and VR24, and the PCR product was cloned into pET30b between XbaI and XhoI, a site 5′ to the His_6 tag, to generate pVR282. To express the SmcR-His_6 protein in *V. vulnificus*, the *smcR*-His tag DNA fragment was amplified from pVR282 by PCR with primers VR22 and VRT7 and then cloned into pJRD215 to generate pVR35. To avoid the degradation of SmcR by the extracellular metalloprotease (Vvp) during purification, pVR35 was introduced into a Vvp-deficient *V. vulnificus* mutant. The Δ*smcR* Δ*vvp V. vulnificus* mutant was isolated by introducing a deletion in *vvp* of CP156 (Δ*smcR* mutant) with pSI026, a suicide plasmid carrying Δ*vvp*, as described previously (26). The resultant strain was grown in LB at 37°C to an OD_{600} of 5, and the bacterial cells were pelleted and then lysed by a French press to release SmcR-His_6 recombinant protein. SmcR-His_6 in the soluble fraction was purified by affinity chromatography with HisTrap HP columns (GE Healthcare). The concentration of purified SmcR-His_6 was estimated by a Bio-Rad protein assay kit.

Gel mobility shift assay. The biotin-labeled probes A, B, and C were amplified from the *hlyU* promoter region by PCR with biotin-labeled primer pairs phlyUF3-phlyURB, phlyUF3B-phlyUR1, and phlyUF4-phlyURB, respectively. Purified SmcR-His_6 was incubated with the probe for 30 min in the presence of 1 μg poly(dI-dC) (used as a nonspecific competitor) as suggested by the manufacturer of the Panomics electrophoretic mobility shift assay (EMSA) kit (Redwood City, CA). The mixture then was fractionated by electrophoresis on a native polyacrylamide gel (6%) and transferred to a membrane, and the probe was visualized by chemiluminescence with the Panomics EMSA kit.

Statistical analysis. The cytotoxicity and cytolysin/hemolysin activity in *V. vulnificus* strains and the levels of β-galactosidase from *lacZ* driven by *vvhBA* promoter in *E. coli* DH5α containing various pVR11.2 derivatives were compared using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test. The mRNA levels and the effect of SmcR on the expression of *hlyU* in various *V. vulnificus* strains were compared using one-way ANOVA with Dunnett's post test and two-way ANOVA with Bonferroni's post test, respectively. All experiments were repeated three times, and the results from a representative experiment were expressed as means ± standard deviations (SD) for each group examined. All tests were performed with GraphPad Prism 4.0, and statistical significance was defined as $P < 0.05$.

RESULTS

Cytotoxicity diminished in Δ*luxO* mutant is restored when *smcR* is further deleted. To investigate the involvement of LuxO in the expression of cytotoxicity in *V. vulnificus*, the cytotoxicity of a Δ*luxO* mutant and a constitutively active *luxO* mutant, *luxO(D47E)*, was assessed. The Δ*luxO* mutant exhibited very low cytotoxicity to HEp-2 cells compared to that of the wild-type strain ($P < 0.001$), while the *luxO(D47E)* mutant was as cytotoxic as the wild-type strain after coincubation for 4 h at an MOI of 10 (Fig. 1A). This indicates that LuxO is associated with the expression of *V. vulnificus* cytotoxicity.

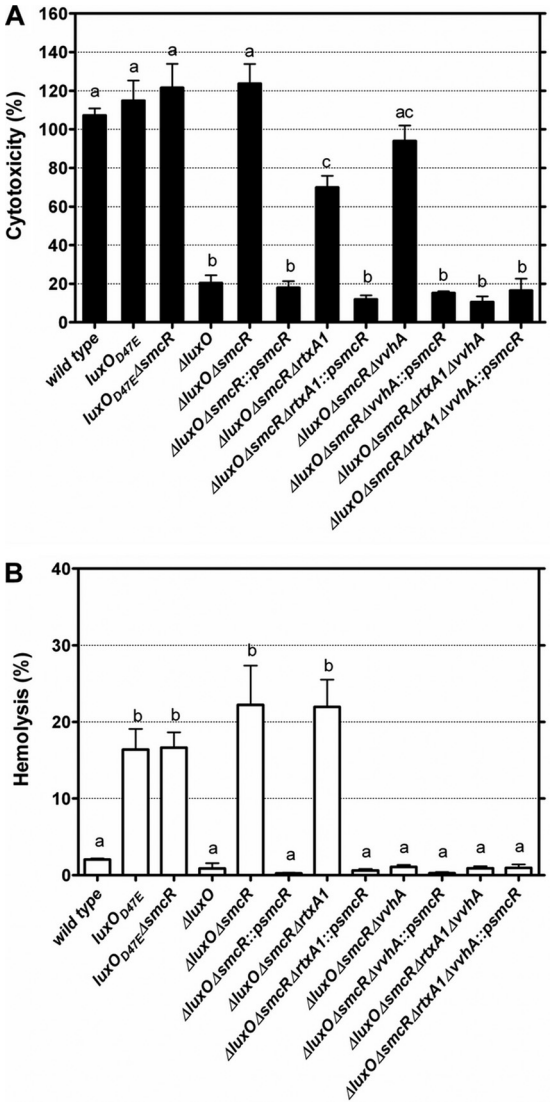

FIG. 1. Cytotoxicity and cytolysin/hemolysin activity of *V. vulnificus luxO* and *smcR* mutants as well as their complemented strains cocultured with HEp-2 cells. The cytotoxicity (A) and cytolysin/hemolysin activity (B) in the conditioned medium were determined after coculture at an MOI of 10 for 4 h. ::*psmcR*, complementation with *smcR*. The bars represent means ± standard deviations; $n = 3$. Bars showing no significant difference are labeled with the same letters, and those showing significant difference ($P < 0.05$) are labeled with different letters based on one-way ANOVA with Tukey's HSD test.

While the expression of SmcR is negatively regulated by LuxO (24), to determine the role of SmcR in the regulation of cytotoxicity by LuxO, we further deleted *smcR* in the Δ*luxO* and *luxO(D47E)* mutants and determined the cytotoxicity of these double mutants. We found that the *luxO(D47E)* Δ*smcR* mutant remained as cytotoxic as the *luxO(D47E)* mutant, but the cytotoxicity of the Δ*luxO* Δ*smcR* mutant, compared to that of the Δ*luxO* mutant, was greatly enhanced ($P < 0.001$) (Fig. 1A). When the Δ*luxO* Δ*smcR* mutant was complemented with *smcR*, the cytotoxicity was reduced to a level comparable to

that of the Δ*luxO* mutant (Fig. 1A). These results suggest that the regulation of cytotoxicity by LuxO is mediated by SmcR, which could repress cytotoxicity.

Cytotoxicity downregulated by *smcR* is attributed to RTX and cytolysin/hemolysin. To determine the contribution of RTX and cytolysin/hemolysin to the cytotoxicity of the Δ*luxO* Δ*smcR* mutant, *vvhA* and *rtxA1* of this mutant were further deleted, and the cytotoxicity of each resultant mutant was measured. The cytotoxicity of the Δ*luxO* Δ*smcR* Δ*rtxA1*, Δ*luxO* Δ*smcR* Δ*vvhA*, and Δ*luxO* Δ*smcR* Δ*vvhA* Δ*rtxA1* mutants to HEp-2 cells was reduced to about 54 ($P < 0.001$), 76 ($P < 0.05$), and 8.5% ($P < 0.01$), respectively, of that of the Δ*luxO* Δ*smcR* mutant (Fig. 1A). The reintroduction of *smcR* into the Δ*luxO* Δ*smcR* Δ*rtxA1* or Δ*luxO* Δ*smcR* Δ*vvhA* mutant resulted in reduced cytotoxicity ($P < 0.01$ and 0.001, respectively) to a level similar to that of the Δ*luxO* Δ*smcR* Δ*vvhA* Δ*rtxA1* mutant (Fig. 1A). However, the reintroduction of *smcR* into the Δ*luxO* Δ*smcR* Δ*vvhA* Δ*rtxA1* mutant did not reduce the cytotoxicity any further (Fig. 1A). These findings suggest that the cytotoxicity downregulated by SmcR is attributed to both cytolysin/hemolysin and RTX.

SmcR downregulates cytolysin/hemolysin production. Mouse RBCs were lysed by treatment with the culture supernatant of a wild-type *V. vulnificus*; however, little hemolysis was detected in the culture supernatant of a cytolysin/hemolysin-deficient mutant (5). This indicates that cytolysin/hemolysin is an extracellular product but RTX is not. Since cytolysin/hemolysin contributed to the cytotoxicity detected in the coculture of bacteria and HEp-2 cells, we determined whether cytolysin/hemolysin was detectable in the conditioned medium by hemolysis assay with mouse RBCs. The wild-type strain exhibited a low level of hemolysis in the conditioned medium, but that of the *luxO(D47E)*, *luxO(D47E)* Δ*smcR*, or Δ*luxO* Δ*smcR* mutant, in which SmcR was either deficient or downregulated, was higher ($P < 0.001$) (Fig. 1B). The deletion of *vvhA*, but not *rtxA1*, in the Δ*luxO* Δ*smcR* mutant abolished the hemolytic activity in conditioned medium (Fig. 1B), showing that indeed cytolysin/hemolysin, and only it, was secreted in the conditioned medium. The hemolytic activity in conditioned medium became almost undetectable when the Δ*luxO* Δ*smcR* and Δ*luxO* Δ*smcR* Δ*rtxA1* mutants were complemented with *smcR* ($P < 0.001$ compared to each parent strain) (Fig. 1B), indicating a negative correlation of *smcR* with the amount of cytolysin/hemolysin secreted.

LuxO and SmcR regulate expression of *rtxA1* and *vvhA* at transcriptional level. To determine if the regulation of cytotoxicity by LuxO and SmcR is through affecting the transcription of *vvhA* and *rtxA1*, the mRNA levels of these two genes in the various *luxO* and *smcR* mutants after coincubation with HEp-2 cells were estimated by real-time reverse transcription-PCR (RT-PCR). As was expected, compared to that of the wild-type strain, the mRNA level of *smcR* was 2-fold increased in the Δ*luxO* mutant ($P < 0.01$), but that in the *luxO(D47E)* mutant was reduced ($P < 0.01$) (Fig. 2A). Meanwhile, the mRNA levels of both *rtxA1* and *vvhA* in the *luxO(D47E)* mutant, in which *smcR* was downregulated, were higher than those in the Δ*luxO* mutant ($P < 0.01$ and 0.05, respectively), in which *smcR* was upregulated (Fig. 2B and C). Nevertheless, when *smcR* in the Δ*luxO* mutant was further deleted, both *rtxA1* and *vvhA* were upregulated ($P < 0.01$) (Fig. 2B and C).

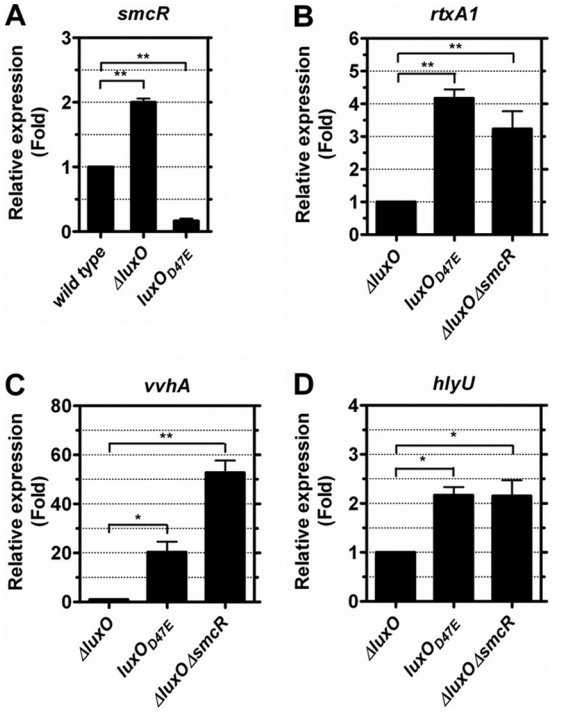

FIG. 2. mRNA levels of *smcR* (A), *rtxA1* (B), *vvhA* (C), and *hlyU* (D) in various *V. vulnificus luxO* and *smcR* mutants after coculture with HEp-2 cells. Washed bacteria prepared from a 4-h culture in LB were coincubated with the HEp-2 cell monolayer at an MOI of 10 for 4 h. The mRNA levels (represented by $2^{-\Delta\Delta CT}$ values) of various genes in the bacteria then were measured by real-time RT-PCR and expressed as the fold change from that measured in the wild-type strain (A) or the Δ*luxO* mutant (B, C, and D). The bar represents means ± standard deviations; $n = 3$. The significance of difference was analyzed by one-way ANOVA with Dunnett's post test. *, $P < 0.05$; **, $P < 0.01$.

Taken together, these data indicate that SmcR negatively regulated the transcription of *rtxA1* and, more evidently, *vvhA*, and in the absence of LuxO, SmcR was upregulated to result in the decreased expression of both *rtxA1* and *vvhA*. In addition, the cytotoxicity to HEp-2 cells and hemolytic activity of the various *luxO* and *smcR* mutants (Fig. 1A and B) correlated well with the mRNA levels of *rtxA1* and *vvhA* (Fig. 2B and C).

HlyU mediates the regulation of *vvhA* by SmcR. To identify the gene(s) that mediates the regulation of *vvhA* by SmcR, we screened a *V. vulnificus* DNA library in an *E. coli* strain, KV372, which contained a transcriptional fusion of the *vvhBA* promoter and *lacZ* (P*vvhBA-lacZ*) as a reporter, for clones showing upregulated P*vvhBA-lacZ* in the presence of SmcR. Three out of approximately 8,000 clones screened exhibited deep blue colonies on the X-gal plates after prolonged incubation. One of them, whose plasmid was designated pVR11.2-L24, contained three intact open reading frames (ORFs), VV0681, VV0682, and VV0683, while the others contained the *lacZ* gene. We further constructed four subclones from pVR11.2-L24 (Fig. 3A) and found that only those (pVR11.2-L243 and pVR11.2-L244) containing ORF VV0683, which encodes HlyU, could upregulate P*vvhBA-lacZ* ($P < 0.001$ compared to the vector control, pVR11.2) (Fig. 3B). When the intact *smcR* gene in pVR11.2-L244 was replaced by Δ*smcR*

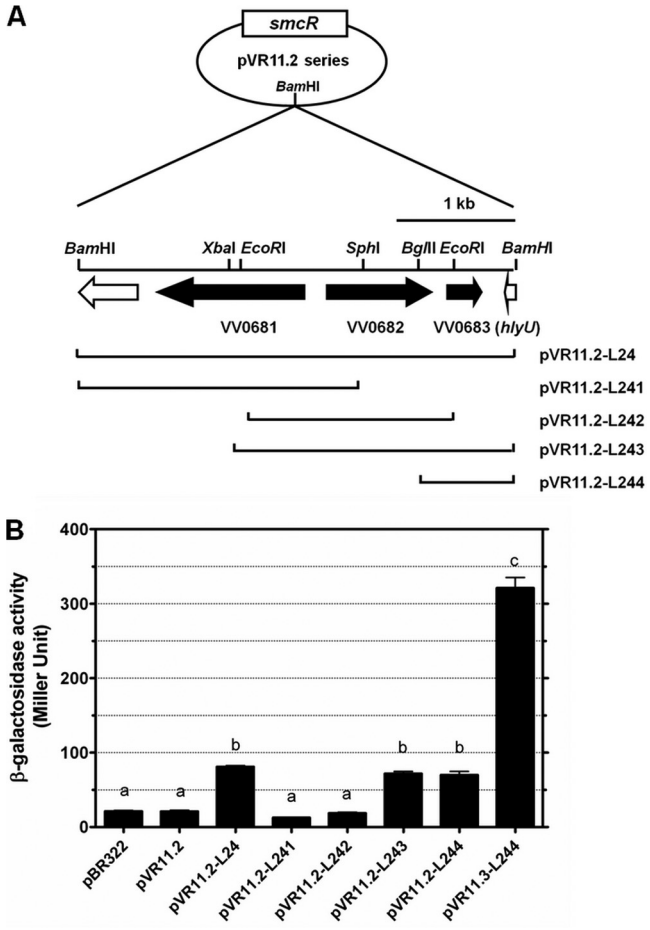

FIG. 3. Identification of regulator of *vvhA*. (A) The restriction map of pVR11.2 series. The three ORFs VV0681, VV0682, and VV0683 (*hlyU*), identified in the insert of pVR11.2-L24, are indicated with black arrows. Plasmids pVR11.2-L241, pVR11.2-L242, pVR11.2-L243, and pVR11.2-L244 are the derivatives of pVR11.2-L24 that contain various parts of the insert as indicated. (B) The levels of β-galactosidase encoded from *lacZ* driven by the promoter of the *vvhBA* operon in *E. coli* DH5α containing various pVR11.2 derivatives. The bacteria were grown in LB at 37°C for 2 h, and the β-galactosidase activity in the culture supernatant was determined. pBR322, vector; pVR11.3-L244, pVR11.2-L244 deleted of *smcR*. Bars represent means ± standard deviations; $n = 3$. Those showing no significant difference are labeled with the same letters, and those showing significant difference ($P < 0.05$) are labeled with different letters based on one-way ANOVA with Tukey's HSD test.

(pVR11.3-L244), the expression of P*vvhBA-lac* was further increased ($P < 0.001$) (Fig. 3B).

***hlyU* is involved in regulation of cytotoxicity by QS signaling.** HlyU has been shown to activate the expression of both *vvhA* and *rtxA1* (19). Consistently with this finding, the cytotoxicity and cytolysin/hemolysin levels of the Δ*hlyU* mutant were abolished ($P < 0.001$ and 0.05, respectively) compared to those of the wild-type strain, and the complementation of the Δ*hlyU* mutant with *hlyU* restored these properties (Fig. 4A and C).

To test whether *hlyU* is associated with the regulation of cytotoxicity and cytolysin/hemolysin production by QS signaling in *V. vulnificus*, this gene in the SmcR-deficient or downregulated mutants was deleted, and the resultant mutants were assayed for these properties. The cytotoxicity of the Δ*smcR*, *luxO(D47E)*, and Δ*luxO* Δ*smcR* mutants was greatly reduced when their *hlyU* gene was further deleted ($P < 0.001$ for all of them) (Fig. 4B). Meanwhile, the hemolytic activity in the conditioned medium of cells infected by these Δ*hlyU* mutants was reduced in all of them ($P < 0.01$, 0.05, and 0.01 for the Δ*smcR* Δ*hlyU*, *luxO(D47E)* Δ*hlyU*, and Δ*luxO* Δ*smcR* Δ*hlyU* mutants, respectively) (Fig. 4D). The complementation of these mutants with *hlyU* restored these properties (Fig. 4B and D).

SmcR downregulates *hlyU*. To test whether *hlyU* is regulated by SmcR, the *hlyU* expression levels in the Δ*luxO*, *luxO(D47E)*, and Δ*luxO* Δ*smcR* mutants after coincubation with HEp-2 cells were estimated by real-time RT-PCR. The transcriptional level of *hlyU* was increased in *luxO(D47E)*, in which *smcR* was downregulated, and Δ*luxO* Δ*smcR* mutants ($P < 0.05$) compared to that of the Δ*luxO* mutant in which *smcR* was upregulated (Fig. 2D).

In another experiment, a DNA fragment containing the intergenic region between VV0682 and *hlyU* was cloned into pVP84 to generate P*hlyU-lacZ* as a reporter, and the resultant plasmid, pVU84, was introduced into the *smcR*$^+$ and Δ*smcR* isogenic strains. Compared to that of the *smcR*$^+$ strain, the Δ*smcR* mutant exhibited higher *hlyU* promoter activity at 3 and 4 h of growth in LB broth ($P < 0.001$ and 0.01, respectively) (Fig. 5A).

SmcR binds to *hlyU* promoter region. A gel mobility shift assay then was performed with the purified His_6-tagged SmcR to determine whether SmcR binds to the promoter of *hlyU*. A *hlyU* promoter-containing DNA fragment (probe A in Fig. 5B) was upshifted in the presence of increasing amounts (from 75 to 300 nM) of SmcR-His_6 (Fig. 5C). The SmcR-binding site was further mapped to the region between −62 and −128 bp (probe B in Fig. 5B) upstream of *hlyU*, as the DNA fragment corresponding to this region caused a band shift but the other fragment (probe C in Fig. 5B), corresponding to a region between +6 and −61, did not (Fig. 5D).

H-NS represses cytotoxicity and cytolysin/hemolysin activity. Recently, ORF VV12923 of *V. vulnificus* strain CMCP6 that encodes an H-NS universal regulator was shown to act as an *rtxA1* repressor and compete with HlyU for binding to the *rtxA1* promoter region (20). To test whether H-NS is a repressor of cytotoxicity and cytolysin/hemolysin production, the *hns* gene (ORF VV1346 in strain YJ016) of the wild-type, Δ*hlyU*, and Δ*smcR* Δ*hlyU* strains was further deleted, and the resultant mutants were characterized. The deletion of *hns* resulted in increases of both the cytotoxicity (except for the wild-type strain, which already showed full cytotoxicity) and cytolysin/hemolysin production in all three strains ($P < 0.001$) (Fig. 6A and B). Moreover, the complementation of these Δ*hns* mutants with *hns* led to a reduction of cytotoxicity (except for the Δ*hns* mutant, which was HlyU proficient) and cytolysin/hemolysin production ($P < 0.001$).

DISCUSSION

In this study, the requirement of the QS regulator, LuxO, in the expression of *V. vulnificus* cytotoxicity was confirmed, and the role of SmcR, a regulator repressed by LuxO (24), in the negative regulation of cytotoxicity was demonstrated (Fig. 1A).

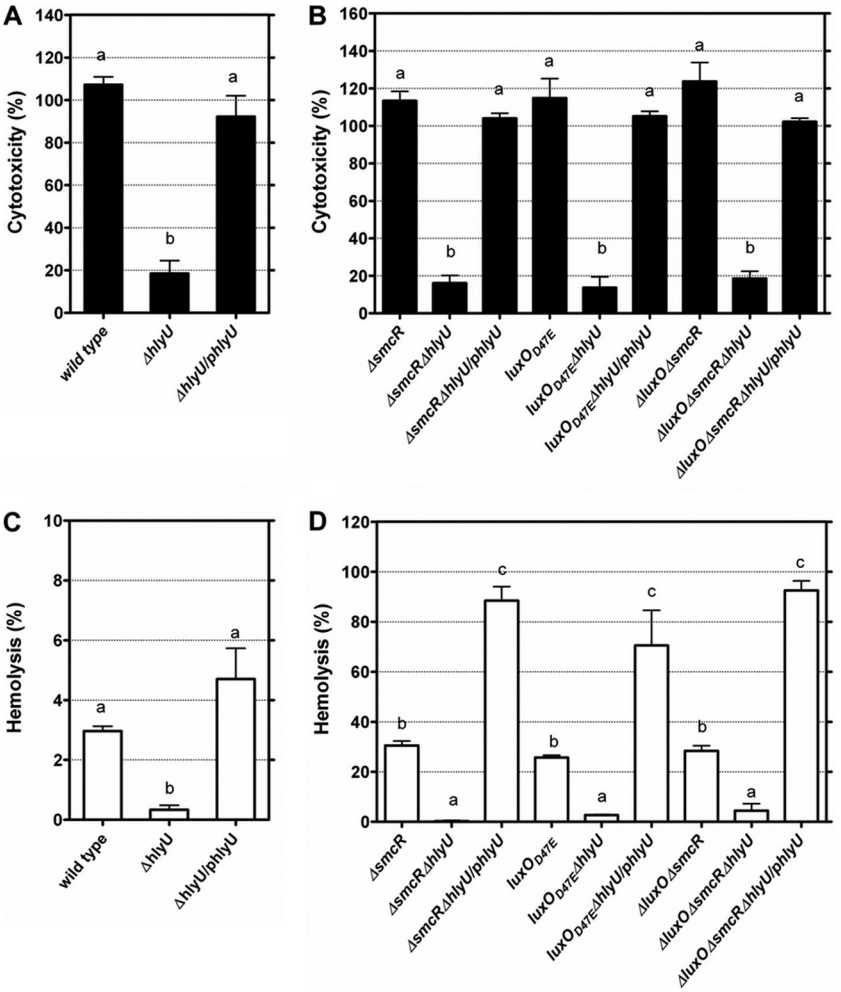

FIG. 4. Cytotoxicity and cytolysin/hemolysin activity of *V. vulnificus hlyU* mutants and complemented strains cocultured with HEp-2 cells. The cytotoxicity (A and B) and cytolysin/hemolysin activity (C and D) in the conditioned medium were determined after coculture at an MOI of 10 for 4 h. */phlyU*, complementation with *hlyU*. The bars represent means ± standard deviations; $n = 3$. Those showing no significant difference are labeled with the same letters, and those showing significant difference ($P < 0.05$) are labeled with different letters based on one-way ANOVA with Tukey's HSD test.

Both RTX and the cytolysin/hemolysin VvhA contributed to the cytotoxicity to HEp-2 cells regulated by QS signaling (Fig. 1A). Further, the transcriptional levels of both *rtxA1* and *vvhA* in the *luxO(D47E)* mutant, which exhibited full cytotoxicity, were significantly higher than those of the Δ*luxO* mutant, which lost cytotoxicity (Fig. 2B and C). These results indicate that the induction of cytotoxicity by QS occurs by activating the transcription of these two cytotoxin genes.

The involvement of RTX in *V. vulnificus* cytotoxicity has been demonstrated, as RTX-deficient mutants exhibit reduced cytotoxicity (15, 19). Several possible mechanisms, including pore formation on the target cell membrane and inducing the apoptosis of the host cells, have been proposed for the cytotoxicity of RTX (12, 13, 15). The Δ*luxO* mutant was shown to be about 100-fold less virulent than the wild-type strain or *luxO(D47E)* mutant, and its spread into the bloodstream was much slower in mice infected subcutaneously (our unpublished data). These phenotypes of the Δ*luxO* mutant are similar to those of an RTX-deficient mutant (15, 19, 21), suggesting a close association of LuxO with RTX-mediated cytotoxicity and virulence in mice.

The role of cytolysin/hemolysin in cytotoxicity is equivocal. Although this protein can cause the cytotoxicity of cultured cells (17, 29), cytolysin/hemolysin-deficient mutants are as cytotoxic as the wild-type strain when they are coincubated with the cells (5, 12, 29). As has been noticed by Kim et al. previously (12), the RTX-deficient mutant still caused cell lysis, and cytolysin/hemolysin was detected in the conditioned medium when it was cocultured with the HEp-2 cells at an MOI of 100 for 4 h (data not shown). The cytotoxicity and cytolysin/hemolysin activity of the RTX-deficient mutants became more evident when cytolysin/hemolysin was overexpressed in mutants deficient in SmcR (Fig. 1A and B). As it has been shown that cytolysin/hemolysin is substantially produced *in vivo* (17), the role of this cytotoxin in pathogenesis needs to be reinvestigated.

We then found that *hlyU*, an activator of both *vvhA* and *rtxA1* (11, 19), mediated the regulation of *vvhA* by SmcR in *E.*

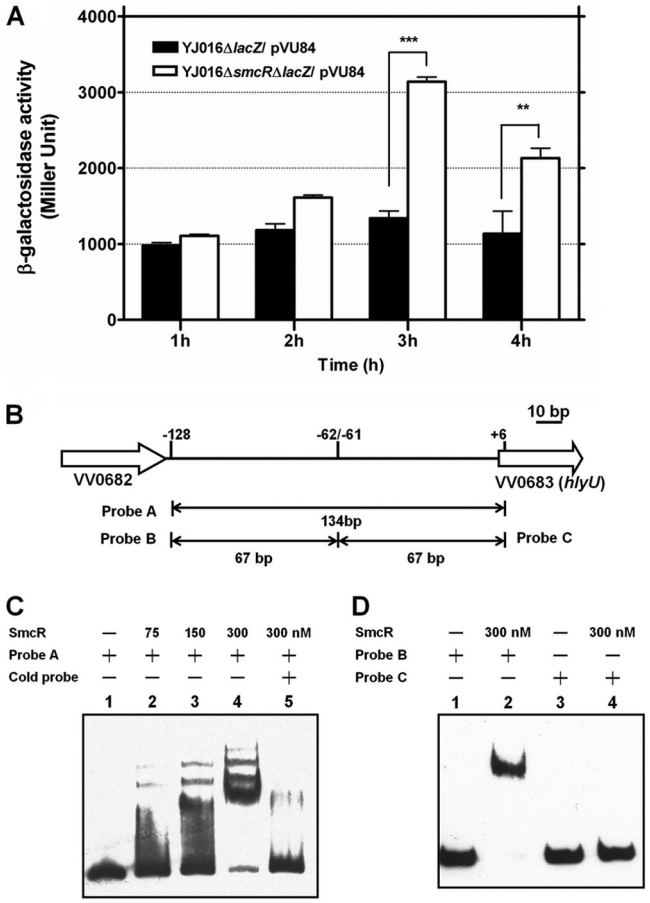

FIG. 5. (A) Effect of SmcR on expression of *hlyU*. The β-galactosidase activity in various *V. vulnificus* strains carrying a P*hlyU-lacZ* fusion in pVU84 was determined after growth in LB for the indicated periods. The bars represent means ± standard deviations; $n = 4$. The significance of difference was analyzed by two-way ANOVA with Bonferroni's post test. **, $P < 0.01$; ***, $P < 0.001$. (B) Organization of VV0682, VV0683 (*hlyU*), and the intergenic region. The regions from which the various probes used in the gel mobility shift assay were derived are indicated. (C) Binding of SmcR to the intergenic region detected by gel mobility shift assay. Lane 1, probe A (2.5 nM) alone; lane 2, probe A plus 75 nM SmcR; lane 3, probe A plus 150 nM SmcR; lane 4, probe A plus 300 nM SmcR; lane 5, probe A plus 300 nM SmcR and 100-fold excess of unlabeled probe A. (D) Binding of SmcR to probes derived from the intergenic region. Lane 1, probe B (2.5 nM) alone; lane 2, probe B plus 300 nM SmcR; lane 3, probe C (2.5 nM) alone; lane 4, probe C plus 300 nM SmcR.

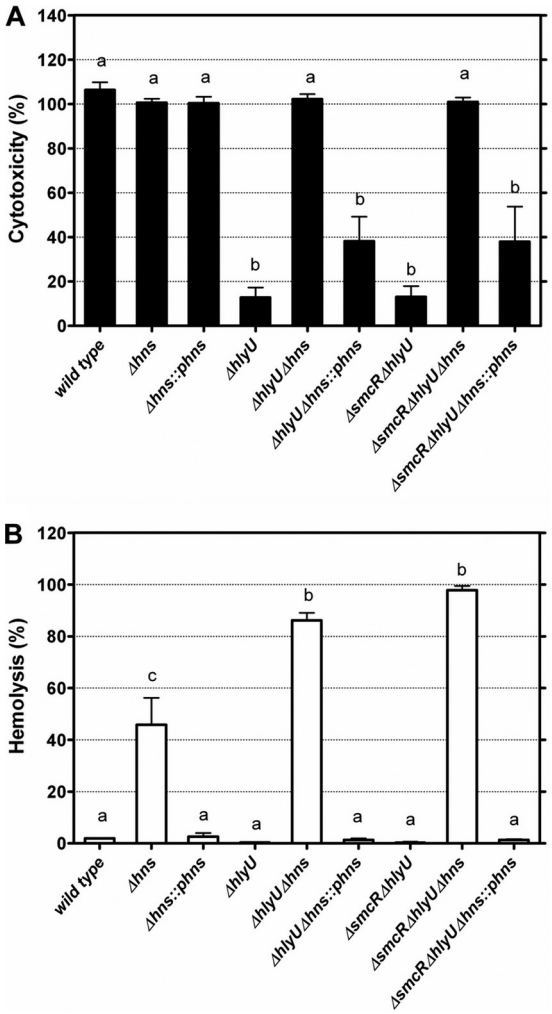

FIG. 6. Cytotoxicity and cytolysin/hemolysin activity of *V. vulnificus* Δ*hns* mutants cocultured with HEp-2 cells. The cytotoxicity (A) and cytolysin/hemolysin activity (B) in the conditioned medium were determined after coculture at an MOI of 10 for 4 h. ::p*hns*, complementation with *hns*. The bars represent means ± standard deviations; $n = 3$. Those showing no significant difference are labeled with the same letters, and those showing significant difference ($P < 0.05$) are labeled with different letters based on one-way ANOVA with Tukey's HSD test.

coli. In *V. vulnificus*, the deletion of *hlyU* in the Δ*smcR* mutant resulted in the abolition of cytolysin/hemolysin activity (Fig. 4B), indicating that HlyU is required for the upregulation of *vvhA* in the Δ*smcR* mutant. We further showed that the promoter activity of *hlyU* was increased in the absence of SmcR (Fig. 5A), and SmcR bound to a region (−128 to −62) upstream of *hlyU* in EMSA (Fig. 5D). In this region we also found a 22-bp sequence (−107 to −86) with 68% (15/22) identity to the consensus SmcR-binding sequence proposed by Lee et al. (14). These findings collectively suggest that SmcR negatively regulates the expression of *vvhA* by repressing *hlyU*. It has been shown that HlyU activates *rtxA1* expression through competition with H-NS, a repressor of *rtxA1*, for binding to the promoter region of *rtxA1* (20). We found in this study that the cytolysin/hemolysin activity in the conditioned medium of cells infected by the Δ*hns*, Δ*hlyU* Δ*hns*, or Δ*smcR* Δ*hlyU* Δ*hns* mutant was significantly higher than those infected by their *hns*$^+$ parent strains (Fig. 6B), suggesting that H-NS also represses the *vvhA* promoter.

As it has been shown that HlyU increases the *rtxA1* promoter activity only in the presence of H-NS (19), we supposed that HlyU regulates the *vvhBA* promoter in a similar manner. Surprisingly, the cytolysin/hemolysin activity of the Δ*hlyU* Δ*hns* or Δ*smcR* Δ*hlyU* Δ*hns* mutant was much higher than, instead of comparable to, that of the Δ*hns* mutant (Fig. 6B). This can be explained by the downregulation of the metalloprotease Vvp in the absence of SmcR (27) or HlyU (11). The cytolysin/hemolysin activity, which declines after late log phase in a wild-type

strain, is sustained in the stationary phase in a Δ*vvp* mutant (26), suggesting that cytolysin/hemolysin is inactivated in the presence of Vvp. Therefore, the higher cytolysin/hemolysin activity in the Δ*hlyU* Δ*hns* or Δ*smcR* Δ*hlyU* Δ*hns* mutant compared to that of the Δ*hns* mutant may be a consequence of reduced Vvp activity.

By epistasis analysis we showed that the reduced cytotoxicity of the Δ*luxO* mutant was restored when *smcR* was further deleted (Fig. 1A), and this double mutant lost cytotoxicity again when its *hlyU* gene was disrupted (Fig. 4A). Furthermore, the cytotoxicity of the Δ*smcR* Δ*hlyU* mutant was regained by deleting the *hns* gene (Fig. 6A). Taken together, how LuxO regulates the cytotoxicity may be delineated: LuxO represses *smcR* to result in the derepression of *hlyU*, and the up-expressed HlyU then interferes with H-NS to cause the derepression of both *vvhA* and *rtxA1*.

Opposite findings on the roles of SmcR in cytotoxicity and virulence in mice have been reported (16). We previously characterized a Δ*smcR* mutant of a clinical isolate, YJ016, and found that both the transcriptional level of *vvhA* and cytolysin/hemolysin activity in this mutant were remarkably increased (26). In addition, the virulence of this mutant in either normal or iron-overloaded C3H/HeN mice challenged by intraperitoneal injection was comparable to that of the wild-type strain. However, an *smcR* mutant derived from another clinical strain, ATCC 29307, exhibited decreased cytotoxicity to INT407 cells and was less virulent in ICR mice (16). These differences may result from the different bacterial strains, cell lines, or mouse strains used, and they also suggest that it is necessary to study the roles of SmcR at different sites in the host, such as the gastrointestinal tract and blood, during infection.

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