THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 46, pp. 34775–34784, November 17, 2006

Transcriptional Regulatory Cascade for Elastase Production in *Vibrio vulnificus*

LuxO ACTIVATES luxT EXPRESSION AND LuxT REPRESSES smcR EXPRESSION[*][S]

Received for publication, August 16, 2006, and in revised form, September 5, 2006 Published, JBC Papers in Press, September 12, 2006, DOI 10.1074/jbc.M607844200

Jong-Bok Roh[‡1], Mi-Ae Lee[‡1], Hyun-Jung Lee[‡], Sung-Min Kim[‡], Yona Cho[‡], You-Jin Kim[§], Yeong-Jae Seok[§], Soon-Jung Park[¶], and Kyu-Ho Lee[2]

From the [‡]Department of Environmental Science, Hankuk University of Foreign Studies, Yongin, Kyunggi-Do 449-791, the [§]Department of Biological Sciences, Seoul National University, Seoul 151 742, and the [¶]Department of Parasitology, The Brain Korea 21 Project, Yonsei University, College of Medicine, Seoul, 133-791, South Korea

Vibrio vulnificus **causes diseases through actions of various virulence factors, including the elastase encoded by the *vvpE* gene. Through transposon mutagenesis of *V. vulnificus*, *vvpE* expression was shown to be increased by *luxO* mutation. Since the *vvpE* gene is known to be positively regulated by SmcR via direct binding to the *vvpE* promoter, the role of LuxO in *smcR* expression was investigated. The *luxAB*-transcriptional fusions containing different lengths of the *smcR* promoter region indicated that the *smcR* transcription was negatively regulated by LuxO and that a specific upstream region of the *smcR* gene was required for this repression. Since LuxO is a known member of positive regulators, the negative regulation of *smcR* transcription by LuxO prompted us to identify the factor(s) linking LuxO and *smcR* transcription. LuxT was isolated in a ligand fishing experiment using the *smcR* upstream region as bait, and *smcR* expression was increased by *luxT* mutation. Recombinant LuxT bound to a specific upstream region of the *smcR* gene, −154 to −129 relative to the *smcR* transcription start site. The expression of *luxT* was positively regulated by LuxO, and the *luxT* promoter region contained a putative LuxO-binding site. Mutagenesis of the LuxO-binding site in the *luxT* promoter region resulted in a loss of transcriptional control by LuxO. Therefore, this study demonstrates a transcriptional regulatory cascade for elastase production, where LuxO activates *luxT* transcription and LuxT represses *smcR* transcription.**

Vibrio vulnificus is a human pathogen that causes fatal septicemia with rapid pathogenic progression and high mortality rates. One of the major virulence factors responsible for this pathology is an extracellular protease called elastase (1, 2), which is a 45-kDa zinc metalloprotease of the thermolysin family and is encoded by *vvpE* (3). VvpE enhances vascular permeability, causes hemorrhagic damage, and degrades type IV collagen in the vascular basement membrane, leading to destruction of the basement membrane and breakdown of capillary vessels (4). Expression of *vvpE* is induced under the conditions at high cell density, and its regulation is mediated by sigma factor S, cAMP-(catabolite regulator protein), and SmcR (5, 6).

SmcR, one of the regulators of *vvpE* expression, is homologous to *Vibrio harveyi* LuxR, which is a master quorum-sensing regulator (7–9). In related pathogens, *Vibrio cholerae*, *Vibrio anguillarum*, and *Vibrio parahemeolyticus*, their virulence factors, such as hemagglutinin/protease, metalloprotease EmpA, and capsular polysaccharide, are regulated by LuxR homologues, HapR, VanT, and OpaR, respectively (10–13). Fine tuning the expression of these virulence factors is achieved by modulation of intracellular levels of this transcriptional factor, LuxR (14). For example, in *V. harveyi*, the *luxR* gene is indirectly repressed by the *luxO* gene product, which is an NtrC-type response regulator (15). Interestingly, LuxR synthesis is regulated at the post-transcriptional level in *V. harveyi* (16). Under low cell density, a phosphorylated form of LuxO activates the transcription of sRNA (16), which destabilizes *luxR* mRNA in the presence of the RNA chaperone, Hfq. Thus LuxO indirectly represses LuxR synthesis. The same mechanism is also operative in repression of *hapR* expression by four sRNAs in *V. cholerae* (16). However, there has been no report yet on the transcriptional control of *luxR*-homologous genes via a cell density-dependent regulatory cascade.

In the present study, we screened a mutant pool of *V. vulnificus* to isolate regulator(s) of extracellular proteases of *V. vulnificus* and obtained a *luxO* mutant. Investigation of the regulatory mechanism explaining the role of LuxO in expression of elastase revealed a transcriptional repressor of *smcR* expression, LuxT, whose expression is activated by LuxO.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for plasmid DNA preparation and for conjugational transfer were grown in Luria-Bertani medium supplemented with appropriate antibiotics at 37 °C. *V. vulnificus* strains were grown in AB medium (300 mM NaCl, 50 mM

* This study was supported by Korea Science and Engineering Foundation, Republic of Korea, Grant R01-2004-000-10046-0 (to S. H. C. and K.-H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ778302.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] These two authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Environmental Science, Hankuk University of Foreign Studies, Wangsan-Li, Mohyun-Myun, Yongin, Kyunggi-Do 449-791, Korea. Tel.: 82-31-330-4039; Fax: 82-31-330-4529; E-mail: khlee@hufs.ac.kr.

$MgSO_4$, 0.2% (w/v) vitamin-free casamino acids, 10 mM potassium phosphate, 1 mM L-arginine, 1% (v/v) glycerol, pH 7.5) (17) at 30 °C, unless stated otherwise. All medium components were purchased from Difco, and the chemicals and antibiotics were from Sigma.

Construction of Deletion Mutants of V. vulnificus—A 1,190-bp DNA containing the *luxO* upstream region was amplified from the genomic DNA of *V. vulnificus* MO6–24/O using two primers, luxO-upF (5′-ATTCGTGACTCGAGGCTAGG-3′; underlined sequence denotes an XhoI restriction site) and luxO-upR (5′-CCTCAAGGGATCCGCTCCGC-3′; underlined sequence denotes a BamHI restriction site). The PCR product was then cloned into pBluescript SK II(+) to produce pSKluxO01. A 592-bp DNA fragment containing the downstream region of the *luxO* gene was made using the primers luxO-downF (5′-ATGGACTTGGATCCGCAAAC-3′; underlined sequence denotes a BamHI restriction site) and luxO-downR (5′-GCTGACATCTAGATAGCCAG-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxO01 to result in pSKluxO02. Then a 1.2-kb kanamycin resistance gene was isolated from pUC4K (Amersham Biosciences) and inserted into the BamHI site of pSKluxO02 to produce pSKluxO03. A 2,982-bp DNA fragment of pSKluxO03 digested with XhoI and XbaI was ligated into a suicide vector, pDM4 (18), to generate pDM4-ΔluxO. The *E. coli* SM10λ*pir* strain carrying pDM4-ΔluxO was conjugated with *V. vulnificus* MO6–24/O, and the exconjugants were then selected on thiosulfate citrate bile sucrose medium supplemented with 4 μg/ml chloramphenicol. Colonies that were positive for double homologous recombination were selected by resistance to 5% (w/v) sucrose, sensitivity to chloramphenicol, and resistance to kanamycin. The selected colony was further confirmed by PCR using primers luxO-upF and luxO-downR and named KPM201.

A 699-bp PCR product containing the *luxT* upstream region was amplified using primers luxT-upF (5′-GGGCCCGTCTGCAACGTCATCGCCTTC-3′; underlined sequence denotes an ApaI restriction site) and luxT-upR (5′-CGGGATCCAGCAACTGATCAACAACGGC-3′; underlined sequence denotes a BamHI restriction site) and then cloned into pBluescript SKII(+) to produce pSKluxT01. A 1,446-bp PCR product was made to contain the downstream region of *luxT* gene using luxT-downF (5′-CGGGATCCAAGCTGGGTTGTTGCGTTGG-3′; underlined sequence denotes a BamHI restriction site) and luxT-downR (5′-GCTCTAGACCATGGCGCTGAATGCACTAC-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxT01 to produce pSKluxT02. *nptI* encoding a kanamycin resistance enzyme was isolated from pUC4K (Amersham Biosciences) and inserted into pSKluxT02 to generate pSKluxT03. The ApaI-XbaI DNA fragment of pSKluxT03 was ligated into pDM4 (18) to produce pDM4-ΔluxT. The resultant plasmid in *E. coli* SM10λ*pir* strain was mobilized to *V. vulnificus* MO6–24/O, and the exconjugants were selected. Colonies with characteristics indicating a double homologous recombination event were isolated as described above (19). Deletion of the *luxT* gene in candidate colonies was confirmed by PCR with primers luxT-upF and luxT-downR and was named SM301.

Azocasein Assay for Exoprotease Activity—Total exoprotease activity of *V. vulnificus* was measured by monitoring the extent of azocasein degradation upon incubation with the spent medium of *V. vulnificus* as described (20). One hundred fifty μl of azocasein solution (20 mg/ml) was mixed with an equal volume of cell-free supernatants of *V. vulnificus* cultures and then incubated at 37 °C for 1 h. The amount of the released azo dye was determined by measuring absorbance at 440 nm with a spectrophotometer.

Zymographic Analysis for Elastase Activity—*V. vulnificus* MO6–24/O and mutant strains were freshly grown in the AB medium at 30 °C for 3 h. The Δ*luxO* mutant strains carrying pRK415-based plasmids were freshly grown in the AB medium supplemented with 3 μg/ml tetracycline at 30 °C for 3.5 h. Twenty μl of each cell-free supernatant, which was mixed with nonreducing Laemmli sample buffer without heat denaturation, was loaded on a 12% (w/v) denaturing polyacrylamide gel copolymerized with 0.3% (w/v) gelatin as described (21). To estimate the protein contents in cell-free supernatants, each sample was treated with 10% (w/v) trichloroacetic acid, and then the total protein amount was measured using a Bradford assay kit (Bio-Rad). An equal amount of protein was used for each cell-free supernatant. Each cell-free supernatant can be seen to include almost the same amount of protein.

Site-directed Mutagenesis of the luxT Promoter—Based on the consensus sequence ($TTGCAN_3TGCAA$) proposed by Lenz *et al.* (16), a putative LuxO-binding site, TTGCACCTAGCAA, was found in the *luxT* promoter region between −312 and −300 relative to the initiation codon of the *luxT* gene. This site was mutagenized using GeneEditor™ *in vitro* site-directed mutagenesis kit (Promega). A DNA fragment containing the *luxT* promoter region in the pLuxT-675 (see the below) was cloned to pGEM-11zf(+) to produce pGEM-11zf(+)-675. Then, luxT-siteF (5′-GTTATCACAGTGAGTGAGCCATGCCTAGCAAGATTTTATAA-3′; underlined bases represent the site for mutagenesis) was used to substitute five bases in the binding site, which resulted in change of TTGCA to CCATG in the putative LuxO-binding site. The resultant plasmid with the mutated LuxO-binding site was named pGEM-11zf(+)-675mt.

Construction of Transcriptional Fusions—To monitor the expression of the *smcR* gene, the *smcR* promoter region was amplified and used to construct transcriptional fusions between the *smcR* promoter and the *luxAB* gene. The promoter region was previously identified and shown to contain a single transcription start site (6). The *smcR* promoter encompassing nucleotides −517 and +126 (relative to the transcriptional start site of the *smcR* gene) was amplified from the genomic DNA of *V. vulnificus* using primers, smcR-nbs (5′-GGGGTACCATTACCGAGCTAGGAAGCCG-3′; underlined sequence denotes a KpnI restriction site) and smcR-down2 (5′-GCTCTAGAAGATAAGCGAGTTCGCGG-3′; underlined sequence denotes an XbaI restriction site). A smaller *smcR* promoter containing nucleotides −48 to +126 (relative to the transcriptional start site of the *smcR* gene) was also amplified by PCR using primers smcR-up3 (5′-GGGGTACCTCACCATAAGTTATTGACCC-3′; underlined sequence denotes a KpnI

TABLE 1
Strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Reference/Source
Strains		
V. vulnificus		
MO6-24/O	Clinical isolate	Ref. 41
QJR70-1	MO6–24/O, *luxO*::mini-*Tn*5 *lacZ*1-Km^R	This study
KPM201	MO6–24/O, Δ*luxO*, Km^R	This study
HS03	ATCC29307, *smcR*, Km^R	Ref. 5
SM301	MO6–24/O, Δ*luxT*, Km^R	This study
KC64	ATCC29307, *vvpE*	Ref. 1
E. coli		
DH5α	f80d*lacZ* DM15 *recA1 endA1 gyrA96 relA1 thi-1 hsdR17*(r_K^- m_K^-) *supE44 deoR* Δ(*lacZYA-argF*)*U169*	Laboratory collection
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::Rp4–2-Tc::Mu λ*pir*;Km^R	Ref. 42
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^-) *relA1 supE44* Δ(*lac-proAB*)[F′*traD3 6proAB lacI*^{q}ZΔM15]	Promega
BMH71-18 *mutS*	*thi supE* Δ(*lac-proAB*), [*mutS*:Tn10] [F′*proAB lacI*^{q}ZΔM15]	Promega
Plasmids		
pHK0011	pRK415 with promoterless *luxAB*, Tc^R	Ref. 22
pHS201	*vvpE*::*luxAB* transcriptional fusion in pHK0011, Tc^R	Ref. 5
pSmcR-517	*smcR*::*luxAB* transcriptional fusion in pHK0011, Tc^R (−517 to +126)	This study
pSmcR-48	*smcR*::*luxAB* transcriptional fusion in pHK0011, Tc^R (−48 to +126)	This study
pGEM-11Zf(+)	General cloning vector, Ap^R	Promega
pGEM-11Zf(+)-675	pGEM-11Zf(+) with upstream region of *luxT*, Ap^R	This study
pGEM-11Zf(+)-675mt	pGEM-11Zf(+)-675, but with mutation in the putative LuxO-binding site, Ap^R	This study
pLuxT-675	*luxT*::*luxAB* transcriptional fusion in pHK0011, Tc^R	This study
pLuxT-675 mt	pLuxT-675, but with mutation in the putative LuxO-binding site, Tc^R	This study
pBluescript II SK(+)	Cloning vector; Ap^R, *lac* promoter (*lacZ*), f1, ColE1	Stratagene
pUC4K	pUC4 with *nptI*, Ap^R, Km^R	Amersham Biosciences
pDM4	Suicide vector; *ori*R6K, Cm^R	Ref. 18
pSKluxO01	pBluescript II SK(+) with 1,190 bp upstream region of *luxO*, Ap^R	This study
pSKluxO02	pSKluxO01 with 592 bp downstream region of *luxO*, Ap^R	This study
pSKluxO03	pSKluxO02 with 1.2-kb *nptI* gene, Ap^R, Km^R	This study
pDM4-ΔluxO	pDM4 containing XhoI and XbaI fragment of pSKluxO03, Cm^R, Km^R	This study
pLAFR5	IncP Tc^R, derivative of pLAFR3 containing a double *cos* cassette	Ref. 43
pLAFR5-luxO	pLAFR5 with 2,135-bp *V. vulnificus luxO*, Tc^R	This study
pSKluxT01	pBluescript II SK(+) with 699-bp upstream region of *luxT*, Ap^R	This study
pSKluxT02	pSKluxT01 with 1,466-bp downstream region of *luxT*, Ap^R	This study
pSKluxT03	pSKluxT02 with 1.2-kb *nptI* gene, Ap^R, Km^R	This study
pDM4-ΔluxT	pDM4 containing ApaI and XbaI fragment of pSKluxT03, Cm^R , Km^R	This study
pQE30	Expression vector, Ap^R	Qiagen
pQE-luxT	pQE30 containing 471-bp *V. vulnificus luxT* coding region, Ap^R	This study

restriction site) and SmcR-down2. Each DNA fragment was digested by KpnI and XbaI, and ligated to KpnI/XbaI-digested pHK0011, which contained the promoterless *luxAB* genes (22). The resultant plasmids, pSmcR-517 and pSmcR-48, were mobilized into *V. vulnificus* MO6–24/O, Δ*luxO* mutant, and Δ*luxT* mutant by conjugation. Exconjugant *V. vulnificus* harboring one of the fusion plasmids were grown in AB medium supplemented with 3 μg/ml tetracycline.

A DNA fragment containing the *luxT* promoter region from −675 to +118 relative to translation initiation codon of the *luxT* gene was amplified from the genomic DNA of wild type *V. vulnificus* using primers luxT-fusF (5′-GGGGTACCTTGGCAAATTCCGCTTGTAGC-3′; underlined sequence denotes a KpnI restriction site) and luxT-fusR (5′-GCTCTAGAGTTGACTCAACGTCGTGTACG-3′; underlined sequence denotes an XbaI restriction site). Another DNA fragment having the same size of *luxT* promoter region, but with a mutagenized LuxO-binding site, was prepared from plasmid pGEM-11zf(+)-675mt using the same primers. Each DNA fragment was digested by KpnI and XbaI and ligated to KpnI/XbaI-digested pHK0011 (22). The resultant plasmids, pLuxT-675 and pLuxT-675mt, have the *luxT* promoter with a wild type LuxO-binding site and mutant LuxO-binding site, respectively. They were mobilized into wild type or Δ*luxO* mutant *V. vulnificus* by conjugation, and the exconjugants were selected in thiosulfate citrate bile sucrose medium supplemented with 3 μg/ml tetracycline.

The light produced by these cells was measured in the presence of 0.006% (v/v) *n*-decylaldehyde using a luminometer (TD-20/20 Luminometer, Turners Designs). Specific bioluminescence was calculated by normalizing the relative light units with respect to cell mass (A_{600}) as described (23).

Western Blot Analysis of SmcR—Cell lysates of wild type, Δ*luxO*, Δ*luxT*, and *smcR V. vulnificus* strains were prepared by sonication in TNT buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20) (24). Eighty μg of each bacterial lysate was fractionated by SDS-PAGE and transferred to a Hybond P membrane (Amersham Biosciences). The membrane was incubated with polyclonal antibodies against SmcR (1:5,000, v/v) and then with alkaline phosphatase-conjugated rabbit anti-rat IgG (1:1,000, v/v; Sigma). Immunoreactive protein bands were visualized using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system (Promega).

Ligand Fishing for Protein(s) Bound to the smcR Promoter—The DNA fragment used as bait was amplified with primers smcR-F1 (5′-biotin-CTAATTCACGAACTCGTTCC-3′) and smcR-R1 (5′-AATGGTGCATATGCATTGGG-3′), which contained the 357-bp promoter region of the *smcR* gene. As a control DNA, the 374-bp coding region of the *smcR* was made with two primers, smcR-F2 (5′-biotin-TTTGCTCGTCGTGGCATTGG-3′) and smcR-R2 (5′-ACTTCACCACGCTCAATGGC-3′). Fifty μg of amplified DNA was loaded onto a NeutraAvidin column as directed by the manufacturer (Pierce). Wild type *V. vulnificus* cells harvested at A_{600} of 0.1 were processed as described previously (25). Proteins eluted from each column were subjected to SDS-PAGE, and the protein bands of interest were excised from the gel and treated as described (25).

The resultant peptides were subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)[3] mass spectrum analysis using a Voyager-DE STR (Applied Biosystems Inc.).

Purification of Recombinant LuxT—Two oligonucleotides, luxT-overF (5′-CGCGGATCCATGCCAAAGCGTAGTAAAGAAGATACC-3′; underlined sequence denotes a BamHI restriction site) and luxT-overR (5′-GGGCTGCAGTATTGGTCGTTATTGACTAATACG-3′; underlined sequence denotes a PstI restriction site), were used to amplify a 471-bp DNA fragment containing the complete open reading frame of the *luxT* gene from the genomic DNA of *V. vulnificus*. BamHI and PstI sites located at both ends of the resultant *luxT* DNA were used to clone this DNA into the pQE30 expression plasmid (Qiagen), to generate a plasmid pQE-luxT. Recombinant LuxT was overexpressed in *E. coli* JM109 by adding isopropyl-thio-β-D-galactoside (Sigma) at a concentration of 1.0 mM, and purified using an Ni^+-nitrilotriacetic acid affinity column as directed by the manufacturer (Qiagen). In the eluted fractions of the Ni^+-nitrilotriacetic acid chromatography, the recombinant LuxT appeared to be a single protein of a high purity, based upon an image of stained protein separated by SDS-PAGE (supplemental Fig. 1).

Gel Shift Assay—Two primers, smcR-comF (5′-CCAAGCTTTCAATACGCAAACGTTCACC-3′) and smcR-down2 (5′-GCTCTAGAAGATAAGCGAGTTCGCGG-3′), were used to amplify a 367-bp fragment of the *smcR* promoter region. The DNA fragment was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and 7 nM was included for each binding assay (24). Binding reactions were carried out in a reaction buffer containing 40 mM HEPES-KOH, pH 7.9, 400 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 10% (v/v) glycerol, and 1 μg of poly(dI-dC) (Sigma). Two different concentrations of recombinant LuxT were used, 100 and 200 nM. The binding mixture incubated for 30 min at 37 °C was then separated on a 6% native polyacrylamide gel. For competition analysis, the same, but unlabeled, *smcR* promoter DNA was added to the binding reaction in a 10- and 30-fold molar excess of the labeled probe. A 378-bp DNA of the *gap* (glyceraldehyde-3-phosphate dehydrogenase) promoter region was amplified from *V. vulnificus* by PCR with primers, gap-F (5′-GGGGTACCGGAATGTAAGCATGCTACCACACC-3′), and gap-R (5′-GGGAATTCCATGGTCTATTCCCTAATGATTCA-3′), and an ~200 nM concentration of this DNA fragment was used as a nonspecific control DNA in the competition experiment.

DNase I Footprinting Assay—A 367-bp DNA fragment of the *smcR* promoter region was amplified by PCR using labeled smcR-down2 primer and unlabeled smcR-comF primer. The binding of recombinant LuxT protein (100, 200, 400, and 800 nM) to the labeled *smcR* promoter (3 nM) was performed for 30 min at 37 °C in a reaction buffer containing 40 mM HEPES-KOH, pH 7.9, 400 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 10% (v/v) glycerol, and 1 μg of poly(dI-dC) (Sigma). The reaction mixture was treated with DNase I for 1 min at room temperature and was terminated with stop buffer (10 mM Tris-HCl, 20 mM NaCl, 20 mM EDTA, 0.2% (w/v) SDS, and 100 μg of tRNA). After precipitation with ethanol, the digested DNA products were resolved by a 4% polyacrylamide sequencing gel alongside sequencing ladders (24). Sequencing ladders were generated from pSmcR-517, a plasmid containing the *smcR* promoter region (Table 1), using labeled smcR-down2 primer and the AccuPower® DNA sequencing kit (Bioneer).

[3] The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

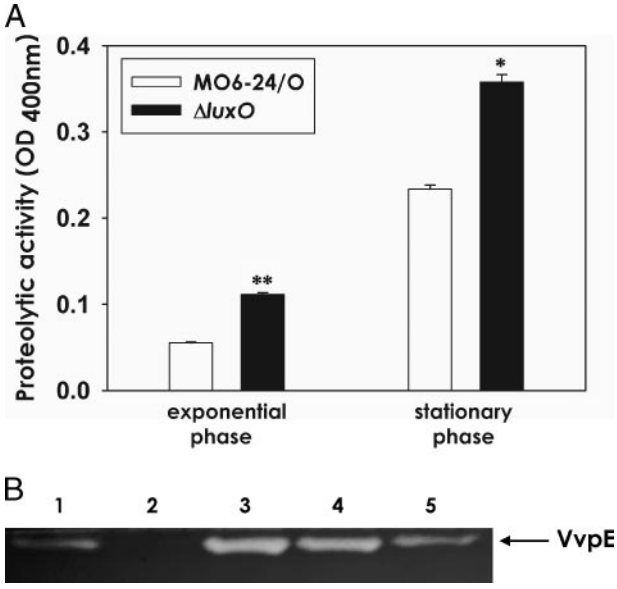

FIGURE 1. **Activity of extracellular proteases produced by wild type and Δ*luxO V. vulnificus*, determined by azocasein degradation analysis (*A*) and zymographic analysis (*B*).** *A*, cell-free supernatants of wild type or Δ*luxO* mutant were sampled at exponential phase (A_{600} of 0.2) and stationary phase (A_{600} of 1.5) and tested for their activity to degrade azocasein. The amounts of the released azo dye due to proteolytic activity of wild type (*open bars*) or Δ*luxO* mutant (*closed bars*) were determined using a spectrophotometer (20). Data with a *p* value of <0.001 (Student's *t* test) are indicated with *two asterisks*, whereas data with a *p* value between 0.001 and 0.01 (Student's *t* test) are represented with an *asterisk*. *B*, the cell-free supernatants of various *V. vulnificus* strains collected at the exponential phase (A_{600} of 0.2) were loaded on a 12% polyacrylamide gel copolymerized with 0.3% (w/v) gelatin. In-gel proteolytic activities of *V. vulnificus* were visualized as clear zones in a gelatin-containing gel upon a Coomassie Brilliant Blue R staining. A clear zone produced by the elastase (VvpE) is indicated with an *arrow*. *Lane 1*, wild type; *lane 2*, elastase-deficient *vvpE* mutant; *lane 3*, Δ*luxO* mutant; *lane 4* Δ*luxO* mutant harboring a broad host range vector, pLAFR5; *lane 5*, Δ*luxO* mutant carrying pLAFR5-*luxO*.

Statistical Analyses—Results were expressed as the mean ± S.D. from three independent experiments. Statistical analysis was performed using Student's *t* test (SYSTAT program, SigmaPlot version 9; Systat Software Inc.). Differences are considered significant if *p* values were <0.01. Data with a *p* value of <0.001 are indicated with two asterisks, whereas data with a *p* value between 0.001 and 0.01 are represented with one asterisk.

RESULTS

Isolation of a Mutant Showing Increased Exoproteolytic Activity—To isolate factors involved in production of exoprotease(s) in *V. vulnificus*, we screened ~10,000 mini-Tn5 *lacZ*1 *V. vulnificus* mutants (26) on agar plates containing 1.5% (w/v) skim milk. One of the mutants, QJR70–1, which showed a distinctively larger clear zone around its colony, was selected as a candidate for increased proteolytic activity. A DNA segment containing the mini-Tn5 was isolated from the genomic DNA of QJR70–1 using the kanamycin-resistant phenotype encoded by mini-Tn5

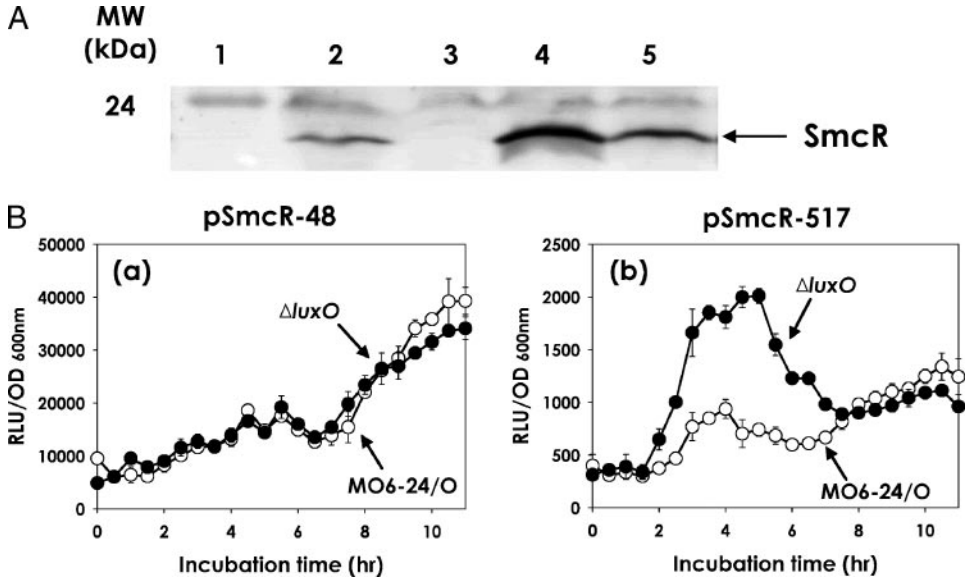

FIGURE 2. **Western blot analysis of SmcR (*A*) and effect of *luxO* mutation on *smcR* expression determined by estimating the expression of *smcR*::*luxAB* transcriptional fusions (*B*).** *A*, cell lysates of the wild type and various mutants collected at the exponential phase were used to estimate cellular contents of SmcR protein. The SmcR protein appeared as an immunoreactive band as indicated with an *arrow*. *Lane 1*, a protein size marker; *lane 2*, wild type; *lane 3*, *smcR* mutant; *lane 4*, Δ*luxO* mutant; *lane 5*, Δ*luxT* mutant. *B*, two *smcR*::*luxAB* transcriptional fusions were constructed. The *smcR* promoter regions used for fusion reporters encompass the DNA region of −517 to +126 and −48 to +126 (relative to the transcriptional start site for the *smcR* gene) (6), and the resultant fusions are designated by pSmcR-517 and pSmcR-48, respectively. Wild type and Δ*luxO* mutant-containing pSmcR-48 (*a*) or pSmcR-517 (*b*) were grown in medium supplemented with 3 μg/ml tetracycline, and aliquots were sampled and determined for their cell masses (A_{600}) and their bioluminescence (relative light units (*RLU*)). Luciferase activities are expressed as normalized values, by dividing the relative light units by the A_{600} of each sample. The activities of three independent experiments were averaged and presented with their S.D. values. The luciferase activities of *smcR*::*luxAB* fusion in wild type *V. vulnificus* are denoted as *open circles*, whereas those in the Δ*luxO* mutant are indicated as *closed circles*.

*lacZ*1 (27). Sequence analysis of the flanking regions of the mini-Tn5 in QJR70–1 revealed that its *luxO* locus was disrupted (data not shown). The *luxO* gene is found to be followed by the *luxU* gene, whose gene product is speculated to be a phosphotransferase in other *Vibrio* spp. (15, 28, 29). The genetic organization of this *luxO-U* cluster is conserved across other *V. vulnificus* strains, YJ016 (GenBank™ accession number NP_933988) and CMCP6 (GenBank™ accession number NP_761887). The deduced amino acid sequence of LuxO of *V. vulnificus* (GenBank™ accession number DQ778302) showed 94, 93, 89, and 75% identity to those of LuxO proteins of *V. parahemeolyticus* (GenBank™ accession number BAC60362), *V. harveyi* (GenBank™ accession number AAD12736), *V. cholerae* (GenBank™ accession number Q9KT84), and *V. fischeri* (GenBank™ accession number YP_204320), respectively.

Generation of a luxO Deletion Mutant and Determination of Its Exoprotease Activities—Since the strain QJR70-1 includes foreign DNA sequence derived from the mini-*Tn*5 *lacZ*1 in its chromosomal DNA, we constructed a *luxO* deletion mutant from the wild type *V. vulnificus* MO6-24/O to exclude any possible effect of transposon DNA on exoprotease activities of *V. vulnificus*. Two sets of primers were used to construct a Δ*luxO* mutant (*i.e.* a set of two primers specific to the upstream region and a second set of primers specific to the downstream region of *luxO*). The resultant Δ*luxO* mutant, KPM201, lost a main portion of the open reading frame of the LuxO protein from amino acid 103 to 239, and instead had the *nptI* gene responsible for resistance to kanamycin. Deletion of the *luxO* gene in chromosome of the mutant *V. vulnificus* was confirmed by PCR using the primers luxO-upF and luxO-downR. The resultant PCR product of the Δ*luxO* mutant *V. vulnificus* appeared to be 3.0 kb, whereas the intact *luxO* gene in the wild type produced a smaller PCR product of 2.2 kb (data not shown).

Cell-free supernatants of both wild type MO6–24/O and Δ*luxO* mutant KPM201 cultures were evaluated for total extracellular protease activity by measuring their ability to degrade azocasein. The total exoprotease activities of the Δ*luxO* mutant were about 2 and 1.5 times higher than those of wild type at the exponential phase and the stationary phase, respectively (Fig. 1*A*). These differences were statistically significant, with $p < 0.001$ during the exponential phase and $p < 0.005$ during the stationary phase.

Since *V. vulnificus* secretes several kinds of exoproteases (1), it was necessary to determine which protease(s) is up-regulated by the *luxO* mutation. Therefore, through a zymographic analysis, exoprotease profiles of wild type and KPM201 were compared with that of an elastase-minus mutant (*vvpE* knock-out mutant) (1). Zymography showed increased elastase activity in the supernatant of KPM201 (Fig. 1*B*). Furthermore, elastase activity returned to normal when the intact *luxO* gene was supplied to the Δ*luxO* mutant using a broad host range vector, pLAFR5, whereas a control plasmid pLAFR5 did not affect elastase activity of the Δ*luxO* mutant. These data suggest that alteration in elastase activity of KPM201 was due to the *luxO* mutation.

To verify that the observed change in elastase activity was attributable to increased *vvpE* expression gene encoding elastase, the expression of *vvpE*::*luxAB* transcriptional fusion (22) was measured in both the wild type and the Δ*luxO* mutant. The *vvpE* expression during the exponential phase increased ~2–3-fold in the Δ*luxO* mutant compared with wild type (data not shown). During the stationary phase, however, there was no difference in *vvpE*::*luxAB* expression between the wild type and the Δ*luxO* mutant (data not shown). The Δ*luxO* mutant showed higher total exoprotease activity than wild type during the stationary phase (Fig. 1*A*), indicating that other exoprotease(s) may be repressed by LuxO in *V. vulnificus*.

Effect of luxO Mutation on smcR Expression—In *V. vulnificus*, expression of the *vvpE* gene is directly controlled by SmcR, a LuxR homologue (5, 6). Therefore, we investigated the mechanism by which LuxO regulates *vvpE* expression and the role of SmcR in this process. Wild type and Δ*luxO* mutant lysates were examined for intracellular SmcR levels by Western blot analysis using polyclonal antibodies that are specific to recombinant SmcR. Δ*luxO* mutant cells con-

tained ~5–6 times more SmcR than wild type cells during exponential phase, based upon densitometric reading of SmcR bands (Fig. 2*A*, *lanes 2* and *4*).

In *V. harveyi*, LuxR is negatively regulated by LuxO at the post-transcriptional level via sRNA and Hfq (16), but there is little information on the transcriptional control of *luxR* by LuxO. Here, we examined the transcriptional effect of LuxO on *smcR* expression by constructing the two *smcR*::*luxAB* transcriptional fusions with different lengths of the *smcR* promoter region (covering −517 to +216 and −48 to +216 nucleotide positions relative to the transcriptional start site for *smcR*). Expression of the shorter fusion (pSmcR-48) was not affected by the mutation at the *luxO* locus (Fig. 2*B*, *a*). The longer fusion (pSmcR-517), however, showed increased expression in Δ*luxO* mutant during the exponential phase (Fig. 2*B*, *b*). This result demonstrated that the LuxO down-regulates *smcR* expression at the transcriptional level during the exponential phase and that repression by LuxO requires the *smcR* upstream region between −517 and −49. The derepressed expression level of pSmcR-517 in Δ*luxO* mutant cells was less than that of pSmcR-48 during exponential phase, suggesting that the *smcR* upstream region may be regulated by factors other than LuxO.

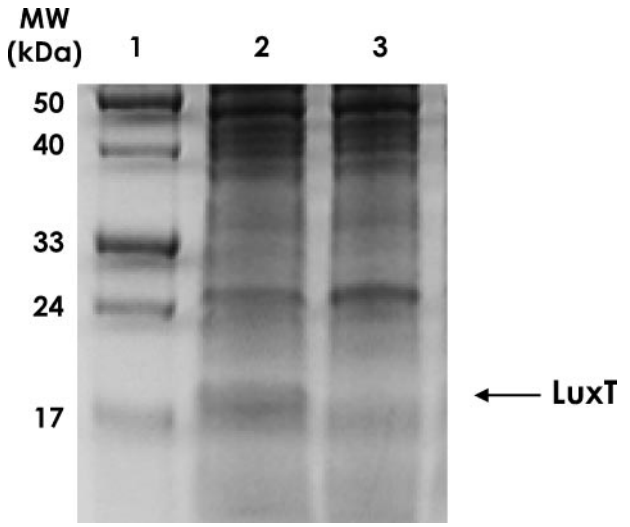

FIGURE 3. **SDS-PAGE of *smcR* promoter-binding proteins retrieved from ligand fishing experiments.** Two DNA fragments, one containing the promoter region of *smcR* (P_{smcR}) and the other encoding the open reading frame of *smcR* (ORF_{smcR}), were amplified by PCR and used as baits for ligand fishing experiments. The proteins bound to P_{smcR} or to ORF_{smcR} were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue R staining. *Lane 1*, protein maker; *lane 2*, proteins bound to P_{smcR}; *lane 3*, proteins bound to ORF_{smcR}. The protein band (designated by an *arrow*), which was specifically bound to the *smcR* promoter, was identified as LuxT by MALDI-TOF mass spectrophotometry.

Isolation of LuxT as a Protein Bound to smcR Promoter Region—Since LuxO is a homolog of NtrC, a well known transcriptional activator (16), the repressive effect of LuxO on *smcR* expression is probably indirect. Therefore, we performed an experiment to isolate the transcriptional factor(s) comprising the regulatory pathway between LuxO and *smcR.* Lysate of *V. vulnificus* was incubated with a DNA fragment containing the *smcR* promoter, and bound proteins were then analyzed by SDS-PAGE. As a control DNA, lysate was incubated with *smcR* coding DNA. One ~18-kDa protein band specifically bound to the *smcR* promoter but not to the *smcR* coding region (Fig. 3). The band, which was excised from the gel and analyzed by MALDI-TOF mass spectrometry, was identified as LuxT (VV21607; GenBank™ accession number NP_367477). LuxT is a member of the TetR family of the transcriptional regulators, which typically repress the target genes (30). The deduced amino acid sequence of *V. vulnificus* LuxT showed 85% identity to those of LuxT proteins of *V. harveyi* (GenBank™ accession number AAK09362) and *V. parahemeolyticus* (GenBank™ accession number NP_799930).

Effect of luxT Mutation on smcR Expression—Our results suggest that LuxO may exert its function as a negative regulator of *smcR* expression through transcriptional activation of *luxT*, which in turn represses *smcR*. To verify the functional role of LuxT in expression of *smcR* of *V. vulnificus*, the *luxT* deletion mutant, SM301, was constructed. Chromosomal deletion of the *luxT* gene was confirmed by PCR using primers luxT-upF and luxT-downR. As expected, the PCR product from the Δ*luxT* mutant with a deletion of the internal region of the *luxT* gene, but with the *nptI* gene instead, was 3.4 kb. Meanwhile, the intact *luxT* in the wild type *V. vulnificus* produced a 2.3-kb PCR product using the same primers (data not shown).

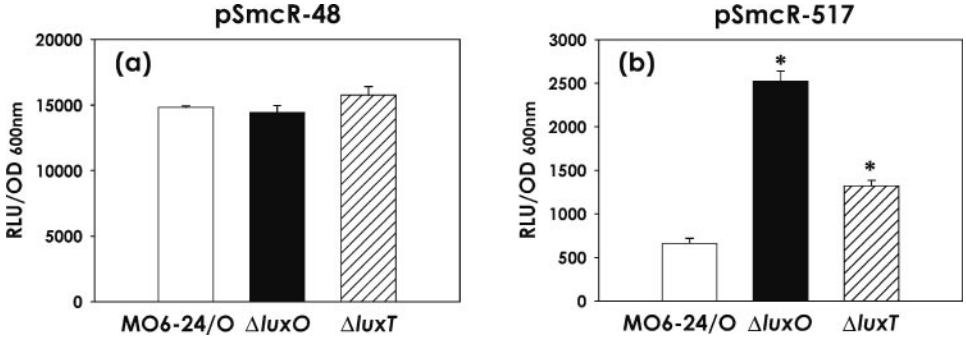

FIGURE 4. **Effect of *luxT* mutation on *smcR* expression determined by estimating the expression of *smcR*::*luxAB* transcriptional fusions.** Expression of two *smcR*::*luxAB* transcriptional fusions, pSmcR-48 (*a*) and pSmcR-517 (*b*), was measured during the exponential phase in Δ*luxT* mutant (*hatched bars*) and compared with those of wild type (*open bars*) and Δ*luxO* mutant (*closed bars*) under the same growth phase. Data marked with an *asterisk* indicate that fusion expression was statistically different from that of wild type cells bearing the same fusion (Student's *t* test; $0.001 < p < 0.01$). Luciferase activities are expressed as normalized values, relative light units (*RLU*) divided by the A_{600} of each sample. The activities of three independent experiments were averaged and presented with their S.D. values.

Western blot analysis of SmcR in the exponential phase *V. vulnificus* cells showed that wild type cells produced a low level of SmcR (Fig. 2*A*, *lane 2*). On the other hand, the Δ*luxT* mutant, SM301, contained ~2–3 times more SmcR protein than wild type (Fig. 2*A*, *lane 5*), based upon densitometric reading of each band. The increase of SmcR in the Δ*luxO* mutant was more distinct than in the Δ*luxT* mutant (Fig. 2*A*, *lane 4*), and the

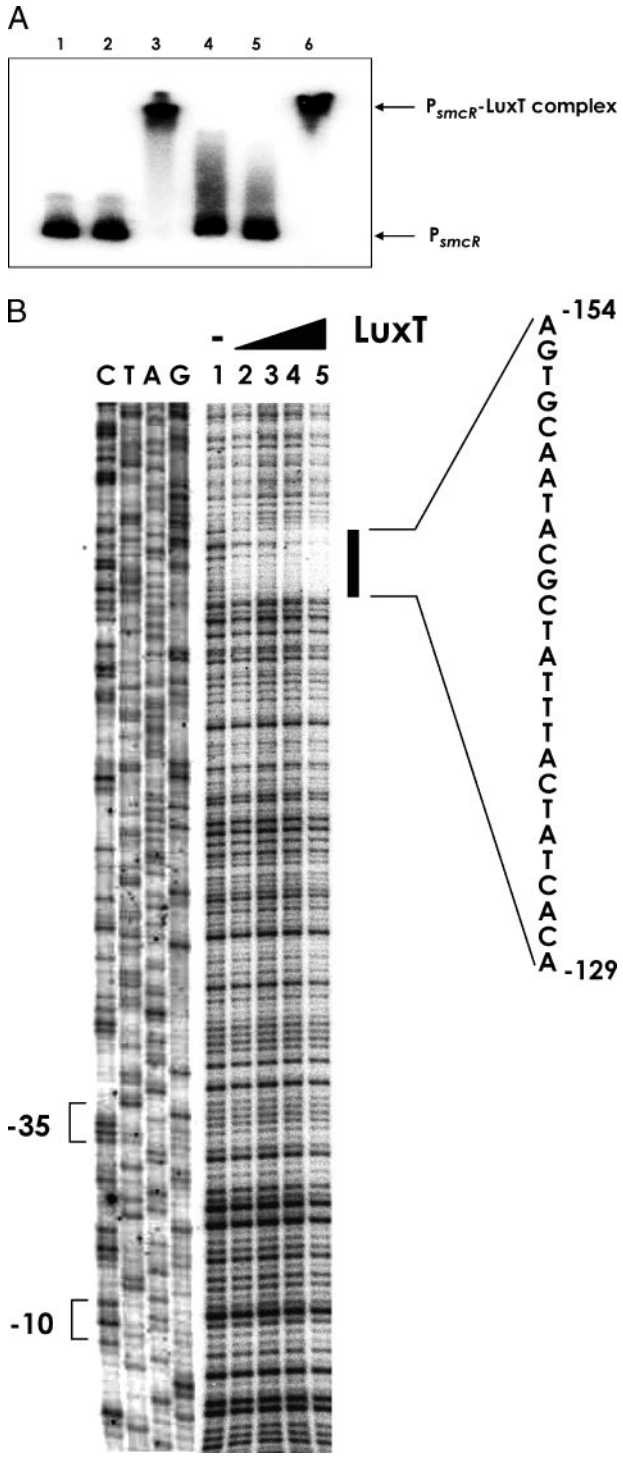

FIGURE 5. **Binding of LuxT to the *smcR* promoter region.** *A*, a gel shift assay was performed to confirm the direct interaction between LuxT and *smcR* promoter region. A ^{32}P-labeled 367-bp DNA fragment of the *smcR* promoter region (P_{smcR}; 7 nM) was mixed with recombinant LuxT. The reaction mixtures were subjected to a 6% native polyacrylamide gel electrophoresis. The slowly moving P_{smcR} was designated as the P_{smcR}-LuxT complex, whereas the free DNA was labeled with P_{smcR}. For competition analysis, the same but unlabeled *smcR* promoter DNA was included in the binding reaction. As a noncompetitive and nonspecific DNA, an unlabeled 378-bp DNA containing the *gap* promoter (P_{gap}) was added to the binding reaction in excess. *Lane 1*, labeled P_{smcR} DNA without LuxT; *lane 2*, labeled P_{smcR} DNA with 100 nM LuxT; *lane 3*, labeled P_{smcR} DNA with 200 nM LuxT; *lane 4*, labeled P_{smcR} DNA with 200 nM LuxT and 70 nM unlabeled P_{smcR} DNA; *lane 5*, labeled P_{smcR} DNA with 200 nM LuxT and 210 nM unlabeled P_{smcR} DNA; *lane 6*, labeled P_{smcR} DNA with 200 nM LuxT and 200 nM unlabeled P_{gap} DNA. *B*, a DNase I footprinting assay was performed to localize the LuxT-binding site in the regulatory region of the *smcR* gene. The ^{32}P-labeled 367-bp DNA fragment of the *smcR* promoter region (3 nM) was incubated with increasing amounts of LuxT protein ranging from 100 to 800 nM, and the reactions were then treated with DNase I. The reaction mixtures were resolved on a 4% polyacrylamide sequencing gel alongside the sequencing ladder derived from the plasmid pSmcR-517. The protected region of the *smcR* promoter was illustrated by a vertical line with the corresponding nucleotide sequences, which are located in nucleotide positions −154 to −129 relative to the transcription start site of the *smcR* gene. *Lane 1*, DNA without LuxT; *lanes 2–5*, DNA with recombinant LuxT protein at 100, 200, 400, and 800 nM, respectively.

level of SmcR in the Δ*luxO* mutant was about 5–6 times higher than in wild type cells during the same growth phase. It may suggest that other factor(s) are involved in the LuxO regulation of *smcR*.

In addition to Western blot analysis, the role of LuxT in *smcR* expression was investigated using the two *smcR*::*luxAB* transcriptional fusions, pSmcR-48 and pSmcR-517. These fusions were introduced into Δ*luxT* mutant, and their luciferase activities were monitored during the exponential phase (Fig. 4). The luciferase activity of pSmcR-48 was not statistically different in Δ*luxT* mutant, Δ*luxO* mutant, or wild type (Fig. 4*a*). In the case of the luciferase activity of the longer fusion, pSmcR-517, it was significantly increased in the Δ*luxT* mutant compared with that of wild type ($p < 0.01$, Student's t test) (Fig. 4*b*). However, the degree of derepression by *luxT* mutation was less than that by *luxO* mutation, since Δ*luxO* mutant showed ~2 times more luciferase activity of pSmcR-517 than Δ*luxT* mutant (Fig. 4*b*). This may imply the presence of LuxT-independent mechanism(s) in the LuxO regulation of *smcR*.

These results suggest that LuxT expression is transcriptionally mediated by LuxO protein during the exponential phase and that LuxT then represses transcription of *smcR*, resulting in reduced production of the elastase. It is also demonstrated that repression by LuxT requires the specific upstream region of *smcR* (−517 to −49 nucleotide position relative to the transcriptional start site of *smcR*).

Specific Binding of LuxT to the smcR Promoter—Gel shift assays were performed to confirm whether LuxT directly binds to the *smcR* promoter region. The 367-bp *smcR* promoter region (which covered from −240 to +126 nucleotide positions relative to the transcriptional start site of *smcR*) was labeled with ^{32}P and incubated with the recombinant LuxT protein (Fig. 5*A*). When the binding reaction was subjected to a native gel electrophoresis, the *smcR* promoter incubated with LuxT at a concentration of 200 nM appeared as a slowly moving band. Specificity of binding was confirmed by a competition experiment using unlabeled *smcR* promoter. The addition of excess unlabeled *smcR* promoter to the binding reaction decreased the interaction between LuxT and the ^{32}P-labeled *smcR* promoter and thus resulted in a disappearance of the slowly moving band. In contrast, the complex formation between LuxT and the labeled *smcR* promoter was maintained, although an excess amount of the *gap* promoter DNA was added to the reaction as a competitor.

To identify the specific LuxT binding site, DNase I footprinting assay was performed. ^{32}P-Labeled *smcR* promoter was incubated with increasing amounts of recombinant LuxT protein, ranging from 100 to 800 nM, and was then treated with DNase I. As a control, labeled *smcR* DNA alone

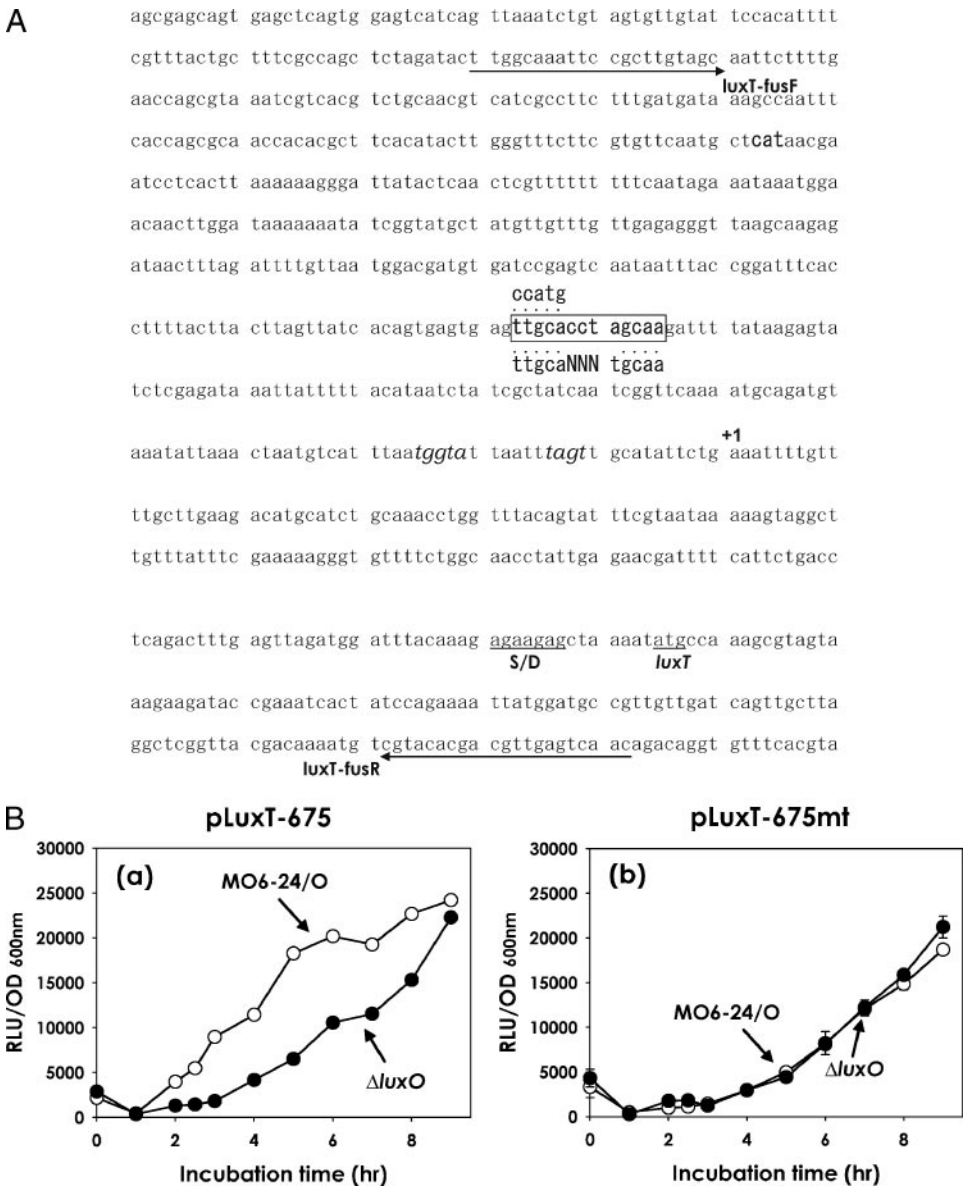

FIGURE 6. **Nucleotide sequence of the upstream region of *luxT* gene (*A*) and expression of *luxT*::*luxAB* transcriptional fusions (*B*).** *A*, the initiation codon and Shine-Dalgarno sequence (*S/D*) of the *luxT* gene are *underlined*. The putative −24/−12 RpoN-binding site is *italicized*, and its putative transcription start site is denoted by +1. The upstream region of the *luxT* gene shows the presence of a putative LuxO-binding site, which was denoted by a *box*. It is highly similar to the consensus sequence, $TTGCAN_3TGCAA$, suggested by Lenz *et al.* (16), and *dots* show the nucleotides that match the consensus sequence. The nucleotides changed in the mutated LuxO-binding sites are shown *above* the putative LuxO-binding site of the *luxT* promoter region. The *horizontal arrows* indicate the positions of two primers used to construct the *luxT*::*luxAB* transcriptional fusion pLuxT-675. The other fusion, pLuxT-675mt, includes the same region of the *luxT* in pLuxT-675 but contains the site-directed mutagenized LuxO-binding site. *B*, the wild type and Δ*luxO* mutant carrying pLuxT-675 (*a*) or pLuxT-675mt (*b*) were grown in medium supplemented with 3 μg/ml tetracycline and measured for their luciferase activity. Luciferase activities of *luxT*::*luxAB* fusions in wild type are denoted as *open circles*, whereas those in the Δ*luxO* mutant are indicated as *closed circles*.

was also treated with DNase I. The DNase I-digested patterns were observed by autoradiography (Fig. 5*B*). When LuxT protein was added to the reaction, a portion of the *smcR* promoter was protected from DNase I, which was located between −154 and −129 (5′-AGTGCAATACGCTATTTACTATCACA-3′) with respect to the transcriptional start site of *smcR*.

Effect of LuxO on luxT Expression—Involvement of LuxO in repression of the *smcR* expression and identification of LuxT as a protein directly bound to the *smcR* promoter led us to determine the hierarchical order of LuxO and LuxT in regulating *smcR* expression. The DNA sequence of the *luxT* upstream region shows the presence of the putative LuxO-binding site ($TTGCAN_3TGCAA$) (16) between −312 and −300 nucleotide positions relative to the translation initiation codon for the *luxT* gene (Fig. 6*A*). The *luxT* upstream region including this site was used to construct a transcriptional fusion with the *luxAB* genes (pLuxT-675), and its expression was monitored in both wild type and Δ*luxO* mutant. Expression of pLuxT-675 was maximal when the bacterial cells entered the stationary phase in both wild type and Δ*luxO* mutant. Its expression in the Δ*luxO* mutant was less than in wild type (Fig. 6*B*, *a*). The *luxO* mutation caused reduction of *luxT*::*luxAB* activity up to one-third of that in wild type cells during the exponential phase.

The role of LuxO as an activator of *luxT* expression was confirmed in an additional experiment using a mutagenized LuxO-binding site. Another *luxT*::*luxAB* transcriptional fusion was made, which included the same *luxT* upstream region as in pLuxT-675 but contained the mutagenized LuxO-binding site (pLuxT-675mt). The degree of expression of pLuxT-675mt in wild type cells was comparable with that in the Δ*luxO* mutant. The expression of the mutagenized *luxT* promoter (pLuxT-675mt) was basically the same as the expression of the intact *luxT* promoter (pLuxT-675) in Δ*luxO* mutant (Fig. 6*B*, *b*). These results indicate that the mutated *luxT* promoter is no longer influenced by LuxO and therefore suggest that LuxO may activate *luxT* transcription by specifically binding to the *luxT* upstream region from −312 to −300.

DISCUSSION

Extracellular enzymes, such as proteases and phospholipases, that are produced by pathogenic bacteria are involved in pathogenesis (2, 31, 32). Zymographic analysis of extracellular proteases secreted from the pathogenic *V. vulnificus* showed that the major proteolytic activity was derived from the elastase

that is encoded by the *vvpE* gene.[4] Despite the ambiguous role of elastase in bacterial toxicity to mice (1), it is able to degrade human vascular basement membrane and capillary vessel (4) and thus is considered as one of the major virulence factors produced by *V. vulnificus* (3). Additionally, elastase production is dependent upon cell density and is controlled by SmcR (22).

There is little information on the quorum-sensing regulation in *V. vulnificus* compared with other *Vibrio* spp. Autoinducer-2 (AI-2) is a quorum-sensing molecule found in *V. vulnificus*, which is able to induce *vvpE* expression (33). The key regulator for *vvpE* expression, SmcR, is a LuxR homolog, which is a well known transcription factor in quorum-sensing control (7, 8). Therefore, elastase production is the only known phenotype regulated by quorum sensing in *V. vulnificus*. In the present study, we screened a mutant pool to isolate regulator(s) for production of exoproteases in *V. vulnificus* and obtained a *luxO* mutant. The finding of LuxO as a regulator for the elastase stimulated us to study the quorum-sensing regulatory cascade in *V. vulnificus* and compare the regulatory characteristics found in other bacteria.

LuxR is a transcription factor that regulates genes related to cell density-dependent phenotypes, such as light production in luminous bacteria and virulence factor production in pathogenic bacteria. Synthesis of this master regulator in *V. harveyi* is regulated by LuxO, which is an NtrC-type response regulator (15). When LuxO is phosphorylated, it becomes active in down-regulation of *luxR* expression. The effect of LuxO on *luxR* expression was assumed to be indirect, since phospho-LuxO acts as a transcriptional activator in conjunction with sigma factor N (RpoN) (34, 35). In *V. harveyi*, binding of sRNA to *luxR* mRNA destabilizes the mRNA, and thus regulation of *luxR* expression occurs at the post-transcriptional level (16). Here, in experiments using *smcR*::*luxAB* transcriptional fusions, LuxO was found to repress *smcR* expression at the transcriptional level in *V. vulnificus* (Fig. 2*B*). Since LuxO putatively activates RpoN-driven transcription, the derepressing effect of *luxO* mutation on *smcR* transcription suggests that LuxO may indirectly regulate *smcR* expression via an unidentified regulator. Therefore, through a ligand fishing experiment, we sought a transcriptional regulator connecting LuxO activation and *smcR* repression (Fig. 3). We identified LuxT as a transcriptional regulator of *smcR* expression in *V. vulnificus*. Expression of the *luxT* gene was activated by LuxO (Fig. 6*B*), and the resultant LuxT repressed the expression of *smcR* gene (Fig. 4b). Thus, these results add LuxT protein to the list of components comprising a regulatory cascade for elastase production (Fig. 7).

Discovery of LuxO as a regulator of *luxT* expression in *V. vulnificus* is interesting, since LuxT has been previously found to regulate the *luxO* expression in *V. harveyi* (36, 37). A genetic approach using site-directed mutagenesis showed that LuxO appeared to directly control the expression of *luxT* in *V. vulnificus*. The putative LuxO-binding site, proposed by Bassler's group (16) is discernable on the *luxT* upstream region (Fig. 6*A*), and mutagenesis of this site abolished the regulatory effect of LuxO (Fig. 6*B*). Therefore, the nucleotide sequences in the *luxT* promoter, TTGCACCTAGCAA (from 312 to 300 bp upstream of the *luxT* gene), might be responsible for binding of LuxO. In addition, *luxT* expression is severely impaired in the *rpoN* mutant *V. vulnificus*.[5]

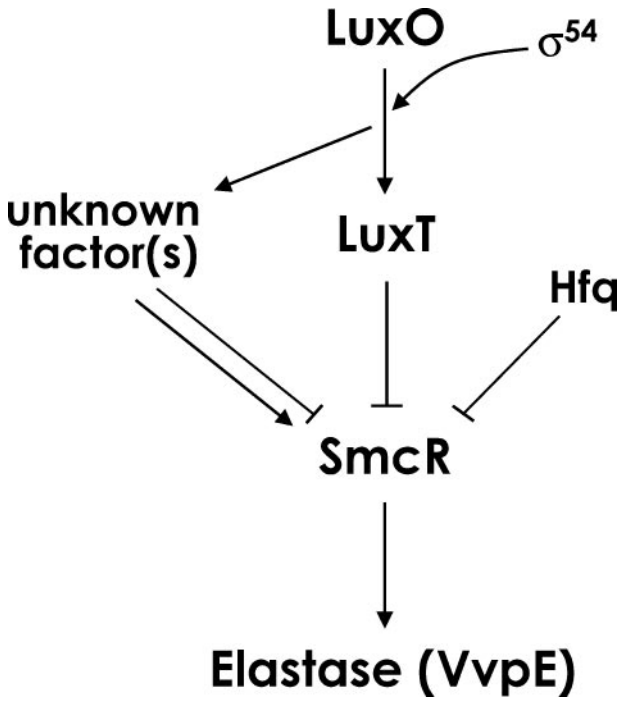

FIGURE 7. **Scheme of the regulatory cascade for elastase production in *V. vulnificus*.** LuxO and sigma factor N (σ^N) activate *luxT* transcription. LuxT represses *smcR* transcription via a direct binding to the upstream region of the *smcR* gene. Then SmcR induces expression of the *vvpE* gene encoding an elastase (5). In addition to involvement of LuxT in regulation of *smcR* expression, *smcR* transcription appears to be regulated by unknown factor(s), which are also induced by LuxO. Hfq negatively regulates *smcR* expression, possibly at the post-transcriptional level with small RNAs (16).

Based on the result of gel shift assays of *smcR* promoter with LuxT protein, we have shown that LuxT plays a role in the expression of *smcR* by directly interacting with the *smcR* promoter (Fig. 5*A*). In addition, the LuxT binding site in the *smcR* promoter region was identified by a DNase I protection assay (Fig. 5*B*), and localized to nucleotides −154 to −129 relative to the transcriptional start site for the *smcR* gene. In *V. harveyi*, LuxT was shown to bind to the *luxO* upstream region, and a potential binding site of LuxT was proposed as a sequence including the repeats of GTT(T/G)A (37). However, we found no such consensus sequence in the region of the *smcR* promoter that was protected by LuxT. Whether the repression of *luxR* genes by LuxT is common in other *Vibrio* species or whether LuxT also regulates *luxO* expression in *V. vulnificus* is unknown. Comparative analyses on the role of LuxT proteins in quorum sensing signal cascade in various *Vibrio* species needs to be done in the future.

The presence of putative sRNA sequences, which showed high similarity to sRNAs found in *V. harveyi* and *V. cholerae*, has been also proposed in *V. vulnificus* (16). In fact, deletion of the *hfq* gene in *V. vulnificus* resulted in increased expression of *smcR*,[6] which suggests that sRNA and Hfq are also involved in *smcR* expression at the post-transcriptional level, as found in

[4] J.-B. Roh and K.-H. Lee, unpublished data.

[5] M.-A. Lee, H.-S. Kim, and K.-H. Lee, unpublished data.

[6] S.-M. Kim, M.-A. Lee, and K.-H. Lee, unpublished data.

V. harveyi and *V. cholerae*. In addition to regulation by LuxT and Hfq, it seems that other transcriptional regulators for *smcR* expression may be present in *V. vulnificus* (Fig. 7). The findings of higher expression of the *smcR* gene in Δ*luxO* mutant than in Δ*luxT* mutant, as determined by Western blot (Fig. 2*A*) and transcriptional fusion assay (Fig. 4*b*), may imply the presence of LuxT-independent mechanism(s) in the LuxO regulation of *smcR*. In *V. cholerae* and *V. anguillarum*, the expression of *hapR* and *vanT* is found to be autoregulated (38, 39). Recently, VqmA protein was found to activate *hapR* expression, but the effect of VqmA on *hapR* is independent of LuxO (40). Therefore, it remains for further studies to elucidate both mechanisms regulating *smcR* expression and roles of the VvpE regulatory cascade for *V. vulnificus* pathogenicity.

Acknowledgments—We thank Kyung-Je Park for constructing the luxO mutant and Sang Ho Choi for generously providing vvpE mutant (KC64) and smcR mutant (HS03).

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J. Biol. Chem. 2006, 281:34775-34784.
doi: 10.1074/jbc.M607844200 originally published online September 12, 2006