Transcriptional Regulatory Cascade for Elastase Production in *Vibrio vulnificus*

**LuxO ACTIVATES luxT EXPRESSION AND LuxT REPRESSES smcR EXPRESSION**

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

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**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 para-hemolyticus*, 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 transcription 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
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MgSO₄, 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′-ATTGCTGACTACGCTAGG-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 luxO was made using the primers luxO-downF (5′-AGGACTTGGATCCGCAAAC-3′; underlined sequence denotes a BamHI restriction site) and luxO-downR (5′-GCTGACATCTGATAGCCG-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxO01 to produce 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 seen to include almost the same amount of protein.

200 μl of each cell-free supernatant, which was mixed with nonreducing Laemmli sample buffer without heat denaturation, was loaded on a 12% (w/v) trichloroacetic acid, and 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 (TTGCAN₃TGCAA) proposed by Lenz et al. (16), a putative LuxO-binding site, TTGCACCTAGC-CAACGTCATCGCCTTC-3′ (underlined sequence denotes a BamHI restriction site), was inserted into the site for mutagenesis. A DNA fragment containing the upstream region of luxO was conjugated with V. vulnificus MO6–24/O, and the exconjugants were selected by resistance to 5% (w/v) sucrose, sensitivity to the light emission, 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.

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.

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′-GGGTTAGCTACGTTACGAGCTGCGGTA-3′; underlined sequence denotes a KpnI restriction site) and smcR-down2 (5′-GTTATACAGTGGATCGCCATTGCTAGAAGAATTTTATAA-3′; underlined bases represent the site for mutagenesis) was used to substitute five bases in the binding site, which resulted in change of TTGCACCTAGC-CAACGTCATCGCCTTC-3′ (underlined sequence denotes an XbaI restriction site) and cloned into pBluescript SK II(+) to produce pSKluxT01. A 1,446-bp PCR product was made to contain the downstream region of luxT gene using luxT-downF (5′-GGGATCCGCTAGGTTGTTGTCGCTG-3′; underlined sequence denotes a BamHI restriction site) and luxT-downR (5′-GCTTCTAGACCATGCCCAGTTACGAAAACTGTTGTT-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxT01 to produce pSKluxT02.

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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 ΔluxO 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.

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MgSO₄, 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.

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A 699-bp PCR product containing the luxT upstream region was amplified using primers luxT-upF (5′-GGGTTACGCTGCAACGTCATC-3′; underlined sequence denotes an Apal restriction site) and luxT-upR (5′-CGGGATCCAGCAACTGATCAACAAAGGC-3′; underlined sequence denotes a BamHI restriction site) and then cloned into pBlue- script SKII(+) to produce pSKluxT01. A 1,446-bp PCR product was made to contain the downstream region of luxT gene using luxT-downF (5′-GGGATCCGCTAGGTTGTTGTCGCTG-3′; underlined sequence denotes a BamHI restriction site) and luxT-downR (5′-GCTTCTAGACCATGCCCAGTTACGAAAACTGTTGTT-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxT01 to produce pSKluxT02. npt II encoding a kanamycin resistance enzyme was isolated from pUC4K (Amersham Biosciences) and inserted into pSKluxT02 to generate pSKluxT03. The Apal-XbaI DNA fragment of pSKluxT03 was ligated into pDM4 (18) to produce pDM4-ΔluxT. The resultant plasmid in E. coli SM10apir 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 named SM301.
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Strains and plasmids used in this study

| Strains/Plasmid | Relevant characteristics | Reference/Source |
|-----------------|--------------------------|------------------|
| **Strains**     |                          |                  |
| V. vulnificus   |                          |                  |
| MO6-24/O        | Clinical isolate         | Ref. 41          |
| QJR70-1         | MO6-24/O, luxO:mini-Tet lacZI-Km<sup>e</sup> | This study       |
| KPM201          | MO6-24/O, luxO<sup>e</sup>, K<sup>m</sup> | This study       |
| HS03            | ATCC29307, smcR<sup>e</sup>, K<sup>m</sup> | Ref. 5           |
| SM301           | MO6-24/O, luxT<sup>e</sup> | This study       |
| KC64            | ATCC29307, vp<sup>g</sup>E | Ref. 1           |
| E. coli DH5a    | b80lacZ DM15 recA1 endA1 gryA96 relA1 thi-1 hsdR17<sup>(r<sub>Km</sub>−, m<sub>Km</sub>−)</sup> supE44 deoR <sup>Δ</sup>[lacZYA-argF-U169]</sup> | Laboratory collection |
| SM10<sub>apur</sub> | thi-1 the leu tona lacY supF <sup>recA4–2–Tc::Mu apirKm</sup> | Ref. 42          |
| JM109           | endA1 recA1 gryA96 thi-1 hsdR17<sup>(r<sub>Km</sub>−, m<sub>Km</sub>−)</sup> relA1 supE44 <sup>Δ</sup>[lac-proAB]F<sup>+</sup>traD3 gprA lacI<sup>Δ26</sup>M<sup>15</sup> | Promega          |
| BHM71-18 mutS   | thi-1 supE<sup>Δ</sup>[lac-proAB], mutS<sup>Tn10</sup> [F<sup>+</sup>proAB lacI<sup>Δ26</sup>M<sup>15</sup>] | Promega          |

**Plasmids**

| Plasmid | Relevant characteristics | Reference/Source |
|---------|--------------------------|------------------|
| pHK011  | pRK415 with promoterless luxAB, Ap<sup>e</sup> | Ref. 22          |
| pHS201  | vp<sup>g</sup>E:luxAB transcriptional fusion in pHK011, Tc<sup>e</sup> | Ref. 5           |
| pSmcR-517 | smcR::luxAB transcriptional fusion in pHK011, Tc<sup>e</sup> | This study       |
| pSmcR-48 | smcR::luxAB transcriptional fusion in pHK011, Tc<sup>e</sup> | This study       |
| pGEM-11Zf(+)-675 | General cloning vector, Ap<sup>e</sup> | Promega          |
| pGEM-11Zf(+)-675mt | pGEM-11Zf<sup>+</sup> with upstream region of luxT, Ap<sup>e</sup> | This study       |
| pLuxT-675 | luxT::luxAB transcription fusion in pHK011, Tc<sup>e</sup> | This study       |
| pLuxT-675 mt | LuxT::luxAB, with mutation in the putative LuxO-binding site, Tc<sup>e</sup> | This study       |
| pBluecript SK(+) | Cloning vector, Ap<sup>e</sup>, lac promoter (lacZ, ft, CoEl) | Stratagene       |
| pUC4K    | pUC4 with nptl, Ap<sup>e</sup>, Km<sup>e</sup> | Amersham Biosciences |
| pDM4     | Suicide vector; oriRbK, Cm<sup>e</sup> | Ref. 18          |
| pSluxK001 | pBluecript II SK<sup>+</sup> with 1.190 bp upstream region of luxO, Ap<sup>e</sup> | This study       |
| pSluxK002 | pSluxK001 with 592 bp downstream region of luxO, Ap<sup>e</sup> | This study       |
| pSluxK003 | pSluxK002 with 1.2 kb gene, Ap<sup>e</sup>, Km<sup>e</sup> | This study       |
| pDM4::LuxO | pDM4 containing Xhol and XbaI fragment of pSluxK003, Cm<sup>e</sup>, Km<sup>e</sup> | This study       |
| pLAFR5   | IncF<sup>Tc</sup>, derivative of pLAFR5 containing a double cos cassette | Ref. 43          |
| pLAFR5<sup>+</sup> | pLAFR5 with 2,135 bp V. vulnificus luxO<sup>e</sup>, Tc<sup>e</sup> | This study       |
| pLuxT01  | pBluecript II SK<sup>+</sup> with 699-bp upstream region of luxT, Ap<sup>e</sup> | This study       |
| pLuxT02  | pSluxK001 with 1.466-bp downstream region of luxT, Ap<sup>e</sup> | This study       |
| pLuxT03  | pLuxT02 with 1.2 kb nptl gene, Ap<sup>e</sup>, Km<sup>e</sup> | This study       |
| pDM4::LuxT | pDM4 containing Apal and Xbal fragment of pSluxK003, Cm<sup>e</sup>, Km<sup>e</sup> | This study       |
| pQE30    | Expression vector, Ap<sup>e</sup> | Qiagen           |
| pQE-LuxT | pQE30 containing 471-bp V. vulnificus luxT coding region, Ap<sup>e</sup> | This study       |

A DNA fragment containing the luxT promoter region from −675 to +118 relative to transcription initiation codon of the luxT gene was amplified from the genomic DNA of wild type V. vulnificus using primers luxT-fusF (5′-GGGGTACCTTGCAAAAT-TCCGCTTGTAGC-3′; underlined sequence denotes a KpnI restriction site) and luxT-fusR (5′-GCTCTAGAGTTGACTG-3′), which was amplified from genomic DNA of wild type V. vulnificus MO6–24/O, ΔluxO mutant, and ΔluxO mutant by conjugation. Exconjugant V. vulnificus harboring one of the fusion plasmids were grown in AB medium supplemented with 3 µg/ml tetracycline.

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Western Blot Analysis of SmcR—Cell lysates of wild type, ΔluxO, ΔluxT, 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 RNA fragment used as bait was amplified with primers smcR-F1 (5′-biotin-CTAATTCCAGAACTCGTCTCC-3′) and smcR-R1 (5′-AATTGGTACATTGATCGTGGG-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-CTTGTCTGTCGACTGGC-3′) and smcR-R2 (5′-ACTTCCACAGCTCAATGGC-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<sub>600</sub> 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).
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The resultant peptides were subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrum analysis using a Voyager-DE STR (Applied Biosystems Inc.).

Purification of Recombinant LuxT—Two oligonucleotides, luxT-overF (5′-CGCGGATCCATGCGTAACTTAACGTAAGATTTAAGAGATACC-3′; underlined sequence denotes a BamHI restriction site) and luxT-overR (5′-GGGCTGCATTTGGCTTATTGCTAATACG-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-β-D-galactoside (Sigma) at a concentration of 1.0 mM, and purified using an Ni2+-nitrilotriacetic acid affinity column as directed by the manufacturer (Qiagen). In the eluted fractions of the Ni2+-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 (supplementary Fig. 1).

Gel Shift Assay—Two primers, smcR-comF (5′-CCAGCTTTCAATGCAAAAAGTTTACC-3′) and smcR-down2 (5′-GCTCTAGAAAGTACCTGGCCTACGGG-3′), were used to amplify a 367-bp fragment of the smcR promoter region. The DNA fragment was labeled with [γ-32P]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 MgCl2, 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 gelatin-containing gel upon a Coomassie Brilliant Blue R staining. A clear zone 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 lacZ1 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.
lacZ1 (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 luxI gene, whose gene product is speculated to be a phosphotransferase in other Vibrio spp. (15, 28, 29). The genetic organization of this luxO-luxI 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. parahaemolyticus (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 Activity—Since the strain QJR70-1 includes foreign DNA sequence derived from the mini-Tn5 lacZ1 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 ΔluxO 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 ΔluxO 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 ΔluxO 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 ΔluxO mutant KPM201 cultures were evaluated for total extracellular protease activity by measuring their ability to degrade azocasein. The total exoprotease activities of the ΔluxO mutant were about 2 and 1.5 times higher than those of wild type at the exponential phase and the stationary phase, respectively (Fig. 1A). 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. 1B). Furthermore, elastase activity returned to normal when the intact luxO gene was supplied to the ΔluxO mutant using a broad host range vector, pLAFR5, whereas a control plasmid pLAFR5 did not affect elastase activity of the ΔluxO 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 ΔluxO mutant. The vvpE expression during the exponential phase increased ∼2–3-fold in the ΔluxO 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 ΔluxO mutant (data not shown). The ΔluxO mutant showed higher total exoprotease activity than wild type during the stationary phase (Fig. 1A), 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 ΔluxO mutant lysates were examined for intracellular SmcR levels by Western blot analysis using polyclonal antibodies that are specific to recombinant SmcR. ΔluxO mutant cells con...
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The longer fusion (pSmcR-517), however, showed increased expression in ΔluxO mutant during the exponential phase (Fig. 2B, 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 ΔluxO 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.

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. parahaemolyticus (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 ΔluxT 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).

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

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

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 spectrometry.

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 ΔluxT mutant (hatched bars) and compared with those of wild type (open bars) and ΔluxO 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.
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level of SmcR in the ∆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 ∆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 ∆luxT mutant, ∆luxO mutant, or wild type (Fig. 4a). In the case of the luciferase activity of the longer fusion, pSmcR-517, it was significantly increased in the ∆luxT mutant compared with that of wild type (p < 0.01, Student’s t test) (Fig. 4b). However, the degree of derepression by luxT mutation was less than that by luxO mutation, since ∆luxO mutant showed ~2 times more luciferase activity of pSmcR-517 than ∆luxT mutant (Fig. 4b).

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 32P and incubated with the recombinant LuxT protein (Fig. 5a). When the binding reaction was subjected to 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 32P-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. 32P-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 performed to localize the LuxT-binding site in the regulatory region of the smcR gene. The 32P-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.

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 32P-labeled 367-bp DNA fragment of the smcR promoter region (PsmcR; 7 nM) was mixed with recombinant LuxT. The reaction mixtures were subjected to a native gel electrophoresis, the smcR promoter DNA was included in the binding reaction. As a non-competitive and nonspecific DNA, an unlabeled 378-bp DNA containing the gap promoter (Pgap) was added to the binding reaction in excess. Lane 1, labeled PsmcR DNA without LuxT; lane 2, labeled PsmcR DNA with 100 nM LuxT; lane 3, labeled PsmcR DNA with 200 nM LuxT; lane 4, labeled PsmcR DNA with 400 nM LuxT; lane 5, labeled PsmcR DNA with 700 nM LuxT and 70 nM unlabeled PsmcR DNA; lane 6, labeled PsmcR DNA with 200 nM LuxT and 210 nM unlabeled PsmcR DNA; lane 7, labeled PsmcR DNA with 200 nM LuxT and 200 nM unlabeled Pgap DNA. B, a DNase I footprinting assay was performed to localize the LuxT-binding site in the regulatory region of the smcR gene. The 32P-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.
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A

The horizontal arrows show the positions relative to the translation initiation codon for the luxT gene (Fig. 6A). 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 ΔluxO mutant. Expression of pLuxT-675 was maximal when the bacterial cells entered the stationary phase in both wild type and ΔluxO mutant. Its expression in the ΔluxO mutant was less than in wild type (Fig. 6B, 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 ΔluxO 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 ΔluxO mutant (Fig. 6B, 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.\textsuperscript{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 \textit{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 \textit{V. vulnificus} compared with other \textit{Vibrio} spp. Autoinducer-2 (AI-2) is a quorum-sensing molecule found in \textit{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 \textit{V. vulnificus}. In the present study, we screened a mutant pool to isolate regulator(s) for production of exoproteases in \textit{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 \textit{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 \textit{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 \textit{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 \textit{V. vulnificus} (Fig. 2B). 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 \textit{V. vulnificus}. Expression of the luxT gene was activated by LuxO (Fig. 6B), 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 \textit{V. vulnificus} is interesting, since LuxT has been previously found to regulate the luxO expression in \textit{V. harveyi} (36, 37). A genetic approach using site-directed mutagenesis showed that LuxO appeared to directly control the expression of luxT in \textit{V. vulnificus}. The putative LuxO-binding site, proposed by Bassler’s group (16) is discernable on the luxT upstream region (Fig. 6A), and mutagenesis of this site abolished the regulatory effect of LuxO (Fig. 6B). 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 \textit{V. vulnificus}.\textsuperscript{5}

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. 5A). In addition, the LuxT binding site in the smcR promoter region was identified by a DNase I protection assay (Fig. 5B), and localized to nucleotides −154 to −129 relative to the transcriptional start site for the smcR gene. In \textit{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 \textit{Vibrio} species or whether LuxT also regulates luxO expression in \textit{V. vulnificus} is unknown. Comparative analyses on the role of LuxT proteins in quorum sensing signal cascade in various \textit{Vibrio} species needs to be done in the future.

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

\textsuperscript{4} J.-B. Roh and K.-H. Lee, unpublished data.

\textsuperscript{5} M.-A. Lee, H.-S. Kim, and K.-H. Lee, unpublished data.

\textsuperscript{6} S.-M. Kim, M.-A. Lee, and K.-H. Lee, unpublished data.
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*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 ΔluxO mutant than in ΔluxT mutant, as determined by Western blot (Fig. 2A) and transcriptional fusion assay (Fig. 4b), 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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