Genes: Structure and Regulation:
Transcriptional Regulatory Cascade for Elastase Production in *Vibrio vulnificus*:
LuxO ACTIVATES luxT EXPRESSION
AND LuxT REPRESSES smcR EXPRESSION

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Transcriptional Regulatory Cascade for Elastase Production in Vibrio vulnificus

LuxO ACTIVATES luxT EXPRESSION AND LuxT REPRESSES smcR EXPRESSION

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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 parahaemolyticus, 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 conjugal 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...
Role of LuxO and LuxT in smcR Expression

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′-ATTCCGTGAATCTAGGCTAGG-3′; underlined sequence denotes an XhoI restriction site) and luxO-upR (5′-CTGCAAGGATGCCGGCTCCGC-3′; underlined sequence denotes a BamHI restriction site) and was named SM301.

The resultant plasmid in E. coli SM10pir strain carrying pBR322::luxO containing the luxO region was subsequently mutagenized using GeneEditorTM in vitro site-directed mutagenesis kit (Promega). A DNA fragment containing the luxO promoter region in the pLuxT-675 (see the below) was cloned to pGEM-11zf(+). The promoter region was amplified from the genomic DNA of V. vulnificus MO6–24/O using primers luxO-upF (5′-ATGAGATGGATCCCGCAAAC-3′; underlined sequence denotes a BamHI restriction site) and luxO-downR (5′-GCTTATCATCAGTAGAGCCG-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxO01 to result in pSKluxO02.

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 ΔluxO mutant strains carrying pRK415::luxO01. A 592-bp DNA fragment containing the downstream region of the luxO gene was used in the primers luxO-downF (5′-ATGAGATGGATCCCGCAAAC-3′; underlined sequence denotes a BamHI restriction site) and luxO-downR (5′-GCTTATCATCAGTAGAGCCG-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxO01 to result in pSKluxO02.

Site-directed Mutagenesis of the luxT Promoter—Based on the consensus sequence (TTGCAN3TGCAA) proposed by Lenz et al. (16), a putative LuxO-binding site, TTGCACCTAGCA, 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 GeneEditorTM in vitro site-directed mutagenesis kit (Promega). A DNA fragment containing the luxT promoter region in the plLuxT-675 (see the below) was cloned to pGEM-11zf(+) to produce pGEM-11zf(+)−675. Then, ΔluxO strain was treated with 10% (v/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.

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 transcriptional 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′-GGGTTAC-CATTACAGGCTAGGAAGC-3′; underlined sequence denotes a KpnI restriction site) and smcR-down2 (5′-GCTTATTGACCC-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxO01 to result in pSKluxO02.

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 transcriptional 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′-GGGTTAC-CATTACAGGCTAGGAAGC-3′; underlined sequence denotes a KpnI restriction site) and smcR-down2 (5′-GCTTATTGACCC-3′; underlined sequence denotes an XbaI restriction site) and cloned into the corresponding sites of pSKluxO01 to result in pSKluxO02.
Role of LuxO and LuxT in smcR Expression

Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains/Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<td>Clinical isolate</td>
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<td>thi-1 leu tona lacY supE recA4 R&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;–</td>
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<td>pHK0011</td>
<td>pRK415 with promoterless luxAB, Tc&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>pH201</td>
<td>vpsE::luxAB transcriptional fusion in pHK0011, Tc&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>pSmcR-517</td>
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<td>This study</td>
</tr>
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<td>pGEM-11Zf&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Promega</td>
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<td>This study</td>
</tr>
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<td>This study</td>
</tr>
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<td>pLuxT&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>This study</td>
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<td>pLuxT&lt;sup&gt;+&lt;/sup&gt;mt</td>
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<td>This study</td>
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<td>pDM4</td>
<td>Suicide vector; oriR5K, Cm&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Ref. 18</td>
</tr>
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<td>This study</td>
</tr>
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</tr>
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<td>This study</td>
</tr>
<tr>
<td>pDM4–luxO</td>
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<td>This study</td>
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</tr>
<tr>
<td>pLAFRS5 luxO</td>
<td>pLAFRS5 with 2,135-bp V. vulnificus luxO, Tc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>This study</td>
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<td>pBluescript II SK&lt;sup&gt;(+)&lt;/sup&gt;</td>
<td>pBluescript II SK&lt;sup&gt;(+)&lt;/sup&gt; with 699-bp upstream region of luxT, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pQE30 containing 471-bp V. vulnificus luxT coding region, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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restriction site) and SmcR-down2. Each DNA fragment was digested by KpnI and Xbal, and ligated to KpnI/Xbal-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, ΔluxO mutant, and ΔluxT 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′−GGGTCATCCGCAATT-TCCGCCTTGATC−3′; underlined sequence denotes a KpnI restriction site) and luxT-fusR (5′−GCCTTAGATGCTACT-TACACGCTGTACG−3′; underlined sequence denotes a Xbal restriction site). Another DNA fragment having the same size of luxT promoter region, but with a mutated LuxO-binding site, was prepared from plasmid pGEM-11Zf<sup>(+)</sup>–675mt using the same primers. Each DNA fragment was digested by KpnI and Xbal and ligated to KpnI/Xbal-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 ΔluxO mutant V. vulnificus by conjugation, and the exconjugants were selected in isothiocyanate 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<sub>600</sub>) as described (23).

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). Immunoreactive protein bands were visualized using the nitro blue tetraazolium/5-bromo-4-chloro-3-indolyl phosphate system (Promega).
Role of LuxO and LuxT in smcR Expression

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′-CGCGGATCCATGCCAAAGCGTAGTAAA-GAAGATACC-3′; underlined sequence denotes a BamHI restriction site) and luxT-overR (5′-GGGCTGCAGATTT-GTGATTATTGACTAATACG-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-ß-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 (supplemental Fig. 1).

Gel Shift Assay—Two primers, smcR-comF (5′-CCAAGC-TTCAATCGAAAAGCTCACC-3′) and smcR-down2 (5′-GCTCTAGAAGATAGCGGGTGCGG-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(dl-dc) (Sigma). Two different concentrations of recombinant LuxT were used, 100 and 200 nM. The binding mixture was incubated for 30 min at 37 °C and 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′-GGGTTAACGGAATGTAAGCAGTACACACC-3′), and gap-R (5′-GGGAATTCAATTGCTATTTGACTAATACG-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 MgCl2, 2 mM dithiothreitol, 10% (v/v) glycerol, and 1 μg of poly(dl-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).

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 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.
Role of LuxO and LuxT in smcR Expression

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-luxU cluster is conserved across other V. vulnificus strains, YJ016 (GenBank™ accession number NP_761887) 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. parahemolyticus (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-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-
Role of LuxO and LuxT in smcR Expression

The longer fusion (pSmcR-517), however, showed increased expression in ∆luxO 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 ∆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.

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; GenBankTM 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 (GenBankTM accession number AA09362) 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 −216 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).

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 A600 of each sample. The activities of three independent experiments were averaged and presented with their S.D. values.

FIGURE 3. SDS-PAGE of smcR promoter-binding proteins retrieved from ligand fishing experiments. Two DNA fragments, one containing the promoter region of smcR (PsmcR) and the other encoding the open reading frame of smcR (ORFsmcR), were amplified by PCR and used as baits for ligand fishing experiments. The proteins bound to PsmcR or to ORFsmcR were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue R staining. Lane 1, protein maker; lane 2, proteins bound to PsmcR; lane 3, proteins bound to ORFsmcR. 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 A600 of each sample. The activities of three independent experiments were averaged and presented with their S.D. values.
Role of LuxO and LuxT in smcR Expression

The level of SmcR in the ΔluxO 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 ΔluxT mutant, and their luciferase activities were monitored during the exponential phase (Fig. 4). The luciferase activity of pSmcR-48 was not statistically different in ΔluxT mutant, ΔluxO 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 ΔluxT 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 ΔluxO mutant showed ~2 times more luciferase activity of pSmcR-517 than ΔluxT 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 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 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 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.
**Role of LuxO and LuxT in smcR Expression**

**A**

The smcR in repression of the transcriptional start site of TATTTACTATCACA-3' which was denoted by a dot. The putative LuxO-binding site of the smcR gene shows the presence of the putative LuxO-binding site. The upstream region of the smcR gene (TTGCAN3TGCAA) (16) between -312 and -300 nucleotide 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 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 (TTGCAN3TGCAA) (16) between -312 and -300 nucleotide 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.

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**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

was also treated with DNase I. The DNase I-digested patterns were observed by autoradiography (Fig. 5B). 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 transcription by specifically binding to the luxT upstream region from -312 to -300.

**FIGURE 6.** Nucleotide sequence of the upstream region of luxT gene (A) and expression of luxT::luxAB transcriptional fusion (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, TTGCAN3TGCAA, 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 ΔluxO 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 ΔluxO mutant are indicated as closed circles.
that is encoded by the vvpE gene. 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. 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 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 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. 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 V. vulnificus.

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 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 consensust 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 need 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 hfg gene in V. vulnificus resulted in increased expression of smcR, which suggests that sRNA and Hfq are also involved in smcR expression at the post-transcriptional level, as found in

Role of LuxO and LuxT in smcR Expression

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.

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