Iron and Quorum Sensing Coordinately Regulate the Expression of Vulnibactin Biosynthesis in Vibrio vulnificus*

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Background: vvsAB are required for the vulnibactin biosynthesis important for the virulence of V. vulnificus.

Results: Fur and SmcR regulate vvsA expression by differential binding to vvsA promoter region in response to iron concentration.

Conclusion: Fur and SmcR regulate vulnibactin synthesis in V. vulnificus, ensuring an appropriate intracellular iron concentration.

Significance: Characterizing the vulnibactin biosynthesis regulation mediated by Fur and SmcR is important to understanding the bacterial pathogenesis.

Vibrio vulnificus is a halophilic marine pathogen associated with human diseases such as septicemia and serious wound infections. Genes vvsA and vvsB, which are co-transcribed and encode a member of the nonribosomal peptide synthase family, are required for vulnibactin biosynthesis in V. vulnificus. In this study, we found that quorum sensing represses the transcription of a vvsAB:lux reporter fusion. Gel shift assay and DNaseI footprinting experiments show that the main regulator of quorum sensing, SmcR, binds to a 22-bp region located between −40 and −19 with respect to the vvsA transcription start site. Mutation of the SmcR binding site abolishes the repression of vvsA:luxAB by SmcR. Fur represses vvsAB transcription in the presence of iron by binding to a 47-bp region located between −45 and +2 with respect to the vvsA transcription start site. A competition gel shift assay and footprinting experiment using Fur and SmcR showed that Fur binds to the vvsA promoter region with higher affinity than SmcR. Studies with the vvsAB::luxAB transcriptional fusion demonstrate that in the presence of iron, Fur is the key repressor of vvsAB transcription, whereas in iron-limited conditions, SmcR is the key regulator repressing vvsAB transcription. This study demonstrates that the Fe–Fur complex and quorum sensing cooperate to repress the transcription of vvsAB in response to iron conditions, suggesting that fine tuning of the intracellular iron level is important for the survival and pathogenicity of V. vulnificus.

Iron is a key element required by bacteria because it plays an important role as a co-factor for several metabolic pathways (1). However, in natural aerobic environments, iron exists in insoluble hydroxyl aquo complexes with a solubility of $10^{-18}$ M at neutral pH (1). Therefore, living organisms generally experi-

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In many bacteria, iron acquisition is regulated by ferric siderophores. The uptake regulator Fur, a transcriptional regulator, is abundant, and Fur is crucial to the regulation of siderophore synthesis. In enteric bacteria, a Fur complex is composed of a Fur protein, which binds to the promoter region of the iron-regulated genes. The Fur-iron complex binds to the promoter region of target genes, and iron is transported into the cell by a specific transport system. The iron-regulated genes are responsible for the synthesis of iron-siderophores, which are transported into the cell by specific transport systems.

Quorum sensing is a process by which microorganisms sense the population of related species. In many bacteria, quorum sensing is involved in the regulation of virulent traits such as toxin secretion. Quorum sensing is mediated by small molecules called autoinducers. In many bacteria, quorum sensing is involved in the regulation of siderophore synthesis. The iron requirements of cells may differ, depending on the cell density. In low cell density conditions, iron is not as important, but at high cell density, SmcR represses the transcription of lucAB.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Condition**—Strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C. *V. vulnificus* strains were grown at 30 °C in LB medium supplemented with 2.0% (w/v) NaCl (LBS) or in AB minimal medium (0.3 M NaCl, 0.05 M MgSO4, 0.2% casamino acids, 10 mM KPO4, 1 mM l-arginine, pH 7.5) (19). All medium components were purchased from Difco, and antibiotics were purchased from Sigma.

**Cloning of smcR and Construction of smcR Deletions in V. vulnificus**—Primers SMCRF01 and SMCRR01 were used for PCR amplification of the DNA fragment of *V. vulnificus* smcR. The iron requirements of cells may differ, depending on the cell density. In low cell density conditions, iron is not as important, but at high cell density, SmcR represses the transcription of lucAB by binding to the promoter region. Meanwhile, under iron-rich condition, Fur binds preferentially to the region and strongly depresses the expression of lucAB, regardless of cell density.
**Regulation of Siderophore Biosynthesis Mediated by Fur and SmcR**

**TABLE 2**

| Name                  | Nucleotide sequence (5′ → 3′)* |
|-----------------------|-------------------------------|
| **smcR knockout mutant construction in ΔluxO and Δfur** |
| KO-SMCUPF             | CACCAGGATGTTTTAACTTGTGGTCAGTAAG |
| KO-SMCRUPR            | CAGAGTCTCGAGAGAATGGATGGGCAGG |
| KO-SMCRDOWNF          | CAATGACTCTAGTTCTTACATCAAGATGT |
| KO-SMCRDOWNR          | AGACTTACGGTCTTTACATCAAGATGT |
| **Complementation of smcR** |
| SMCRF01               | TGTTTCTTAAAGAGAAACATATAAGCAC |
| SMCRR01               | CAAGAATCCTCGTTGTATATGTTCTG |
| **Construction of vvsA::luxAB fusion** |
| FS-VVS.AF01           | GGGGTACACCCAATAGGACAGGAGAAG |
| FS-VVS.R01            | GCCCTTAAAGAGAAACATATAAGAC |
| **Real-time PCR**     |
| RT-VVS.AF02           | CTTCCCTTGCCTTTATTTGCCCA |
| RT-VVS.R02            | TGGCTCTTTCCCAACAGCTTCTA |
| RT-SMCRF01            | CAATGPTTTCTGCAGACGCTTCTT |
| RT-SMCR.R01           | GCTTTGAGTTTGGATGTTCTT |
| RT-RPSL.F01           | AACCAGTGTGATGACGAGCAGG |
| RT-RPSL.R01           | CCTTACCCGAGTTTGATGATG |
| **Protein purification** |
| STREP-SMC.RF          | CCGGAATTCCTGCAGAGCGTTCG |
| STREP-SMC.RR          | CCGGAATTCCTGCAGAGCGTTCG |
| STREP-FURF            | GTATGAATTCATGTCAGACAATAC |
| STREP-FRR             | QVTACTCGAGTTATCTGACAGG |
| **Primer extension**  |
| PE-VVS.AF06           | CTTCCCTTAAAGAGAAACATATAAGC |
| **Gel shift assay and DNaseI footprinting** |
| EMSA-VVS.AF03         | TCTTTCTCCAGGGAGCAGGAGG |
| EMSA-VVS.R03          | CAACGAACCTTGTGAAGAAAGAGG |
| EMSA-RPSL.F02         | CCGCAGACCTGGAAGCAGG |
| EMSA-RPSL.R02         | TTAAGACTTTAGAGCTTACACG |
| **Site-directed mutagenesis** |
| MT-VVS.AF04           | CTGAGTTTAAAGAAGCCAGAGGTCAAGTATGTTATATTAC |
| MT-VVS.R04            | GATATAAATATTTACATCRTCAGGCTTATTTTAAATCTAGG |
| MT-VVS.AF05           | GATACCCCTAGTTAGTATAGAAGG |
| MT-VVS.R05            | TCTGACGACAGCTCTTAGGAAG |

* Nucleotides modified for the generation of a restriction site or site-directed mutagenesis are underlined.

The resulting product was cloned into pRK415 (20) to construct pRK415-smcR. A DNA fragment comprising the upstream region of smcR was amplified using primers KO-SMCRUPF and KO-SMCRUPR and ligated to the pGEM-T Easy vector (Promega, Madison, WI), generating plasmid pGEM-T-SMCRup. The downstream region of smcR was amplified using primers KO-SMCRDOWNF and KO-SMCRDOWNR and ligated to the pGEM-T Easy vector, generating plasmid pGEM-T-SMCRdown. Plasmid pGEM-T-SMCRup was digested with restriction enzymes PstI and BamHI, and the resulting DNA fragment containing the smcR upstream region was ligated to plasmid pGEM-T-SMCRdown. Plasmid pGEM-T-SMCRup was digested with restriction enzymes PstI and BamHI, and the resulting DNA fragment containing the smcR upstream region was ligated to plasmid pGEM-T-SMCRdown to construct pGEM-T-SMCRKO. pGEM-T-SMCRKO was digested with XhoI and BglII and ligated to pDM4 (21) to obtain pDM4-SMCRKO, which was then introduced into E. coli S17-1 ppir to be mobilized into V. vulnificus ΔluxO and Δfur by conjugation. Double crossover selection to construct the chromosomal deletion of smcR was performed using 10% sucrose LB agar plate as described previously (21). The chromosomal smcR deletion mutations were confirmed by PCR and DNA sequencing.

**Detection of Siderophore Production**—V. vulnificus MO6-24/O was cultured overnight in LB medium and then washed and subcultured in AB minimal medium at 30 °C until stationary phase (A<sub>600</sub> of ~1.0). Siderophore production was quantitatively measured using the universal assay described by Schwyn and Neilands (22). Briefly, 0.5 ml of culture supernatant and 0.5 ml of Chrome Azurol S solution were mixed. After the addition of 10 μl of shuttle solution (0.2 M 5-sulfsalicylic acid), the mixture was incubated for 10 min at room temperature. Siderophore production was quantified by measuring the absorbance at 630 nm (23). Siderophore activity was expressed as relative siderophore units (RSU) normalized to cell density: 100 × (A<sub>630</sub> of AB medium control − A<sub>630</sub> of V. vulnificus culture supernatant)/A<sub>600</sub> of cell culture.

**Purification of Fur and SmcR Proteins**—A DNA fragment encoding 205 amino acids of SmcR was PCR-amplified using primers STREP-SMC.RF and STREP-SMC.RR (Table 2). The amplified fragment was subcloned into pASK-IBA-7 (IBA, Göttingen, Germany), which results in an SmcR fusion protein with a Strep tag at the N terminus. The resulting construct was transformed into E. coli BL21 (DE3) (Novagen, Madison, WI), and expression of the Strep-tagged SmcR was induced with 0.2 μg/ml anhydrotetracycline solution. After centrifugation, bacterial pellets were suspended in buffer W (100 mM Tris-Cl, 150 mM NaCl, pH 8.0), sonicated, and then centrifuged at 13,000 rpm for 15 min. The resulting supernatant was applied to 1 ml of Strep-Tactin-Sepharose resin (IBA), and specifically bound proteins were eluted with buffer E (100 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8.0). The eluted protein was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel to assess purity. Fur was purified using a similar approach. The DNA sequence encoding 149 amino acids of Fur was PCR-amplified using primers STREP-FUR-F and STREP-
Regulation of Siderophore Biosynthesis Mediated by Fur and SmcR

FURR (Table 2). The amplified fragment was subcloned into pASK-IBA-7, and Strep-tagged Fur was purified as described above.

Preparation of Polyclonal Rabbit Antibsera against Purified Fur and Western Blot Hybridization—Purified Fur was used for the production of polyclonal rabbit antisera (Ab Frontier, Seoul, Korea). For Fur expression analysis, overnight cultures of V. vulnificus MO6-24/O wild type, ΔluxO, ΔsmcR, ΔluxOΔsmcR, and Δfur were washed and subcultured in AB minimal medium until late log phase (A600 of ~0.6). Cells were washed, and 20 μg of each lysate was resolved by SDS-PAGE and transferred to a Hybond P membrane (GE Healthcare). The membrane was incubated with polyclonal rabbit antisera against Fur (1:2000), and subsequently with goat anti-rabbit IgG-HRP (1:2000) (Santa Cruz Biotechnology). Fur expression was visualized using Western blotting Luminol reagent (Santa Cruz Biotechnology).

Construction of vvsA::luxAB Transcriptional Fusion—Primers FS-VVSAR01 and FS-VVSAR02 were used for PCR amplification of the vvsA promoter region from −632 to +121 relative to the transcription initiation site. The resulting product was digested with KpnI and XbaI and cloned into pHK0011 (24) to construct pHVSAA632, which was then conjugated into V. vulnificus MO6-24/O wild type, ΔluxO, ΔsmcR, and ΔluxOΔsmcR strains.

Luciferase Assay—Overnight cultures of V. vulnificus strains were washed with AB medium and inoculated into AB medium containing 2 μg/ml tetracycline. Samples were diluted 25-fold with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4, pH 7.4). After adding 0.006% (v/v) n-decylaldehyde, luminescence was measured using a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany). Specific transcriptional level was expressed as the light units normalized to cell density (RLU) as described previously (25).

Determination of the vvsA Transcription Start Site by Primer Extension—RNA was extracted from wild-type strain MO6-24/O cultured in AB minimal medium supplemented with or without 25 μM FeCl3 grown to an A600 of approximately 1.0. Primer PE-VVSA06, which is complementary to the 5’-end of vvsA, was 32P-labeled and used to synthesize first-strand cDNA from 1 μg of RNA using SuperScript III reverse transcriptase (Invitrogen). The same primer was used to generate a sequencing ladder using a Top DNA Sequencing Kit (Bioneer, Seoul, Korea). The resulting primer extension product and sequencing ladder were resolved in a 6% polyacrylamide sequencing gel. The gel was dried and read using BAS-1500 Imaging Plate (Fujifilm, Tokyo, Japan).

Gel Shift Assay—A 280-bp DNA fragment containing the upstream region of the vvsA promoter (nucleotide +90 to −190 with respect to the transcription start site of vvsA) was PCR-amplified using 32P-labeled primers EMSA-VVSA03 and EMSA-VVSA03 (Table 2). For gel shift assays, 8 nM labeled DNA fragment was incubated with increasing amounts of purified SmcR (0–1 μM) or Fur (0–1 μM) in a 20-μl reaction for 30 min at 37 °C. The SmcR binding reaction contained 10 mM HEPES, 100 mM KCl, 200 μM EDTA, and 10% glycerol, pH 7.5. The Fur binding reaction contained 10 mM HEPES, 100 mM KCl, and 10% glycerol, pH 7.5, supplemented with 100 μM MnSO4 or 1 mM EDTA. The binding reaction was terminated by the addition of 3 μl of loading buffer, and samples were resolved in a 6% neutral polyacrylamide gel. For the Fur and SmcR competition gel shift assay, 8 nM labeled DNA was incubated with different amounts of purified SmcR and Fur in the presence of 1 mM EDTA or 100 μM MnSO4, and samples were processed as described above.

Site-directed Mutagenesis of the SmcR Binding Region

Upstream from vvsA—A DNA fragment containing the vvsA promoter region (−168 to +108 relative to the transcription start site) was PCR-amplified using primers MT-VVSAF05 and MT-VVSA05 (Table 2). The resulting product was digested to pGEM-T Easy vector to construct pGEM-TvvsA. The SmcR binding site was mutated using primers MT-VVSAF04 and MT-VVSA04 and the Quick Change® Site-directed Mutagenesis Kit (Strategene). The resulting plasmids, pGEM-TvvsAmt and pGEM-TvvsA, were both digested with restriction enzymes KpnI and XbaI and ligated into pHK0011 to construct pHVSAA168 and pHVSAA168mt, respectively. pHVSAA168 and pHVSAA168mt were introduced into wild-type V. vulnificus MO6-24/O, ΔluxO, and ΔluxOΔsmcR strains by bi-parental mating using S17-1 as donor (26). Exconjugants were selected on thiosulfate–citrate–bile salts–sucrose agar (TCBS) plates (Difco) supplemented with 2 μg/ml tetracycline. Luciferase activity was measured as described above.

DNaseI Footprinting Assay—An end-labeled 348-bp DNA fragment of the vvsA promoter regions (−160 to +180 relative to the transcription initiation site) was amplified using primers EMSA-VVSA03 and 32P-labeled EMSA-VVSA03. To determine the SmcR binding site, 200 ng of the amplified vvsA promoter region was incubated with increasing amounts of purified SmcR at 37 ºC for 30 min in 50 μl of buffer (10 mM HEPES, 100 mM KCl, 200 μM EDTA, 10% glycerol, pH 7.5). To identify the Fur binding site, 200 ng of the amplified vvsA promoter region was incubated for 30 min with increasing amounts of purified Fur in 50 μl of binding solution (10 mM HEPES, 100 mM KCl, 10% glycerol, 100 μM MnSO4, pH 7.5). After 30 min, 50 μl of CaCl2-MgCl2 solution (10 mM MgCl2, 5 mM CaCl2) was added to the binding reaction. DNasel was then added (0.25 unit, Promega), and the reaction was incubated at 37 ºC for 1 min. The reaction was terminated by the addition of 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS). After addition of 500 μl of ethanol, samples were precipitated on ice for >30 min and centrifuged. DNA pellets were washed with 70% ethanol and resuspended in 10 μl of loading buffer (0.1 M NaOH:formamide (1:2), 0.1% xylene cyanol, 0.1% bromphenol blue). The samples and the sequencing ladder generated with 32P-labeled EMSA-VVSA03 were denatured for 5 min at 95 ºC, chilled on ice, and loaded on to a 6% sequencing gel.

To investigate binding competition between Fur and SmcR on the vvsA promoter region via a footprinting assay, 1 μM Fur and/or 1 μM SmcR was incubated with 200 ng of the 348-bp DNA probe described above in 50 μl of buffer (10 mM HEPES, 100 mM KCl, 10% glycerol, increasing concentrations of MnSO4 or EDTA, pH 7.5) for 30 min. Samples were processed as described above.
Quantitative Real-time PCR (RT-PCR) Analysis—RNA was isolated from *V. vulnificus* using the RNeasy® Mini Kit and the RNase-free DNase Set (Qiagen, Valencia, CA). Purified RNA was quantified using a Biophotometer (Eppendorf, Hamburg, Germany). cDNA was synthesized from 1 µg of RNA using PrimeScript™ RT reagent kit (Takara, Ohtsu, Japan). The reaction was incubated at 37 °C for 30 min, and 2 µl of cDNA was subjected to RT-PCR analysis on an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA), using SYBR® Premix Ex Taq™ (Takara, Ohtsu, Japan). RT-PCR was performed in a 96-well plate (Bio-Rad) in triplicate in a 25-µl volume. The *rpsL* gene was used as an endogenous loading control for the reactions. The amount of transcript was analyzed with 7500 SDS Software (Applied Biosystems).

**RESULTS**

SmcR Represses Siderophore Production at the Transcription Level—We have observed that production of siderophore in *V. vulnificus* MO6-24/O diminished as the cells entered the stationary phase of growth (8). This observation suggested that siderophore production is regulated by cell density, prompting us to study the effect of quorum sensing on siderophore production. We constructed chromosomal deletion mutations in smcR, the main regulatory protein for quorum-sensing signaling in *V. vulnificus*, and luxO, the key regulator that represses the expression of smcR (17). As shown in Fig. 1A, siderophore synthesis in wild-type bacteria is approximately 2-fold higher than in the *luxO* mutant and lower than in the *smcR* mutant and *luxO smcR* double mutant. Introduction of a wild-type *smcR* plasmid into the *smcR* mutant strain restored the siderophore level to level similar to that of the *luxO* mutant. The effects of these mutations on the transcription of *vvsA* were evaluated using a *luxAB* reporter transcriptionally fused to *vvsA*. As shown in Fig. 1B, in the wild-type strain, the transcription of *vvsA-LuxAB* was greatest as cells reached the exponential phase of growth and started to diminish in early stationary phase. In contrast, in the *luxO* deletion mutant, *vvsA* expression was reduced to about half the level of that in wild-type bacteria. In the *smcR* mutant, *vvsA* expression was maintained at a high level even in stationary phase. These results suggest that expression of *vvsA* is regulated by quorum sensing. Siderophore activity and *vvsA* expression in the *smcR* mutant were almost the same as in the *luxO* double mutant (Fig. 1B), suggesting that the signal to SmcR was delivered directly from LuxO via quorum sensing and not from any other regulatory pathways. Introduction of a wild-type *smcR* plasmid complemented the defect in *vvsA* transcription in the *smcR* mutant, as assessed by RT-PCR (Fig. 1C). These results confirm that the reduced level of siderophore production in stationary phase is due to the repression of transcription by the SmcR-mediated quorum-sensing regulatory pathway.

**FIGURE 1. Quorum sensing regulates siderophore biosynthesis in *V. vulnificus*.** A, siderophore production in *V. vulnificus* MO6-24/O wild type, ΔluxO, ΔsmcR, ΔluxOSmcR, ΔsmcR harboring pRK415, and ΔsmcR harboring pRK415-smcR. RSU refers to siderophore unit/µg cells. Data are the average values from three independent experiments, and error bars denote S.D. B, luciferase activities from *vvsA-luxAB* transcriptional fusion harbored by *V. vulnificus* MO6-24/O wild-type (filled circles), ΔluxO (filled squares), ΔsmcR (open triangles), and ΔluxOSmcR (open squares). RLU (relative light units) refers to luciferase activity normalized by A600. Data are the average values from three independent experiments, and error bars denote S.D. C, RT-PCR of the *vvsA* transcript in *V. vulnificus* ΔsmcR harboring pRK415 or pRK415-smcR. RNA levels were quantified by using the comparative threshold cycle (ΔΔCt) method, and RNA-fold change was normalized to the value in ΔsmcR harboring pRK415-smcR. The data are average values from three independent samples, and error bars denote S.D.
Iron Represses vvsA Transcription in the Presence of Fur

It is well known that the Fur is a global regulator of genes involved in iron acquisition in other bacteria (3). To investigate whether Fur regulates genes for biosynthesis of siderophore in V. vulnificus, transcription of the vvsA::luxAB fusion was measured with or without exogenous iron. As shown in Fig. 2, in the wild-type strain, the addition of iron highly repressed vvsA transcription, and depletion of iron derepressed vvsA transcription. In contrast, in the fur mutant, transcription of vvsA was completely derepressed regardless of the iron concentration (Fig. 3A), demonstrating that Fur can tightly repress the transcription of vvsA.

Fur Is Not Regulated by SmcR—The observation that both Fur and the quorum-sensing regulator SmcR were associated with the regulation of vvsA led us to hypothesize that the effects of SmcR on siderophore production might be mediated through SmcR regulation of Fur, in the presence of iron. To test this possibility, the expression of Fur was assessed in wild-type V. vulnificus and in isogenic smcR and luxO mutants by Western blot hybridization using polyclonal rabbit antisera against Fur. As shown in Fig. 3A, the ΔfurΔsmcR, ΔluxOΔsmcR, and ΔfurΔluxOΔsmcR strains showed no significant differences from the wild-type strain in the levels of Fur, indicating that SmcR does not affect the expression of Fur. RT-PCR analysis of fur transcript levels also showed no differences between these strains (data not shown). These data indicate that SmcR does not affect vvsA expression via modulation of Fur expression but rather directly regulates vvsA transcription through a Fur-independent pathway. To verify this, derivatives of fur smcR double mutant harboring a pRK415-smcR or pRK415 were examined for vvsA

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expression in iron-limited media. SmcR repressed the *vvsA* expression in the absence of *fur* (Fig. 3B), demonstrating that under low iron conditions, *smcR* regulates *vvsA* expression independently of *fur*.

**Localization of the vvsA Transcription Start Site**—Primer extension analysis was performed to determine the transcription start site of *vvsA*. As shown in Fig. 4, primer extension reveals that the transcriptional initiation site of *vvsA* (indicated by the asterisk in Fig. 4) is located 20 bp upstream of the translation initiation codon. The amount of the transcript was qualitatively reduced with iron supplementation (compare lanes 1 and 2 in Fig. 4), further demonstrating that the transcription of *vvsA* is negatively controlled by iron at the transcriptional level.

**SmcR Represses vvsA by Direct Binding to the vvsA Promoter Region**—After excluding a role for Fur in SmcR-dependent regulation of *vssAB* (above), we hypothesized that SmcR may regulate the *vvsA* transcription via direct binding to a *cis*-element in the *vvsA* promoter region. To test this hypothesis, a gel shift

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**FIGURE 5. Binding of SmcR to the promoter region of vvsA.** A, gel shift of the labeled 280-bp *vvsA* promoter region by purified SmcR. Lanes 1-5 are SmcR concentrations 0, 50, 100, 200, and 300 nM, respectively. Lanes 6 and 7 are 30 and 100 nM concentrations, respectively, of the same DNA fragment, unlabeled, incubated with 300 nM SmcR and 8 nM labeled DNA fragment. B, gel shift of radiolabeled *rpsL* probe as a negative control incubated with increasing amounts of SmcR. Lanes 1-5 are SmcR concentrations 0 nM, 125 nM, 250 nM, 500 nM, and 1 µM, respectively. C, DNaseI protection of the region upstream of *vvsA* by SmcR. Lanes 1–5 are 0, 125 nM, 250 nM, 500 nM, and 1 µM SmcR incubated with 200 ng of labeled 348-bp *vvsA* promoter region, respectively. Lanes T, C, G, and A represent the corresponding sequencing ladder generated with 32P-labeled EMSA-VVSAR03. Nucleotide sequences protected by SmcR are highlighted in black.
assay was performed with purified SmcR and the vvsA promoter region. A 280-bp end-labeled DNA fragment including the putative vvsA promoter region was incubated with increasing amounts of purified SmcR. As shown in Fig. 5A, SmcR was able to bind to the vvsA promoter region and was outcompeted by an excess of unlabeled probe. A negative control labeled probe amplified from the rpsL gene encoding 30 S ribosomal protein S12 (27) was not bound by SmcR (Fig. 5B), indicating that binding of SmcR to the vvsA promoter is specific.

A 22-bp consensus sequence for SmcR binding has been proposed based on examination of promoter regions of V. vulnificus genes directly regulated by SmcR (5’-TTATTGATWWR-WTWNTAAATAA-3’; W represents A or T, R represents G or A, N represents any nucleotide) (18). However, a similar sequence is not apparent in the vvsA promoter region. To identify the binding site of SmcR in the vvsA promoter region, DNasel footprinting was performed. A 22-bp region spanning −40 to −19 from the transcription initiation site of vvsA was protected from DNasel digestion by SmcR (Fig. 5C). This SmcR binding sequence exhibits an inverted repeat (5’-AAATGATGTTAATGATAATTATT-3’, inverted repeat bases are italicized) that might form a hairpin structure.

To confirm that the binding site is essential for SmcR regulation of vvsA transcription, 7 bases of the SmcR binding sequence were mutagenized, including changes that would disrupt the formation of a putative hairpin structure (Fig. 6A). Transcriptional luxAB reporter fusions to the wild-type vvsA promoter region and the mutated vvsA promoter regions with altered SmcR binding sequences were constructed and named pHVVSA168 and pHVVSA168mt, respectively. The constructs were introduced into the ΔluxO strain, in which smcR is constitutively expressed, and into the ΔluxOΔsmcR strain, and the expression of vvsA was measured. The wild-type promoter fusion (pHVVSA168) showed depressed vvsA transcription in ΔluxO compared with ΔluxOΔsmcR, whereas the fusion with the mutagenized SmcR binding site (pHVVSA168mt) showed similar derepressed expression in both the ΔluxO and ΔluxOΔsmcR strains (Fig. 6B). These results indicate that the SmcR binding site we identified is an essential cis-acting element for SmcR regulation of vvsA transcription.

Fur Binds Specifically to the Promoter Region of vvsA—It is well known that Fur regulates iron acquisition genes by binding to the fur box in the presence of iron. The vvsA promoter includes a putative 19-bp fur box (5’-GTTAATGATAATTATC-3’) with only two nucleotide differences from the consensus fur box (5’-GATAATGATAATCATTATC-3’) of E. coli (28). To determine whether Fur binds to the region upstream of vvsA, a gel shift assay was performed using purified Fur protein. In the presence of 100 μM manganese, which is a typical substitute for ferrous iron in in vitro binding reactions (29), Fur binds to the promoter region of vvsA specifically (Fig. 7A). In contrast, when EDTA was added to chelate the free divalent ion, Fur was not able to bind to region upstream of vvsA (Fig. 7B). To identify the binding site of Fur, DNasel footprinting analysis was performed using purified Fur in the presence of manganese (Fig. 7C). The 47-bp DNA region protected by Fur lies between −45 and +2 relative to the vvsA transcription initiation site.

Fur and SmcR Bind Differently in Response to Divalent Metal Ion Conditions—The genetic organization of the vvsA promoter region is summarized in Fig. 8. The region protected by SmcR overlaps that protected by Fur, suggesting that the two might compete for the shared binding region. The putative −10 promoter box also overlaps with the Fur and SmcR binding
sites, suggesting that they inhibit transcription by interfering with the binding of RNA polymerase. These overlapping binding regions led us to investigate how these two regulatory factors compete for binding to the vvsA promoter. In vitro competition gel shift assays with either purified Fur or SmcR showed that, in the presence of EDTA, the 280-bp vvsA promoter fragment described under “Experimental Procedures” was bound by SmcR, and binding of Fur was hardly detected even at high Fur concentration (up to 300 nM) (Fig. 9A). However, when manganese was supplied in the absence of EDTA, Fur bound well, and SmcR did not (Fig. 9B). When Fur and SmcR were simultaneously incubated with the promoter fragment, Fur preferentially bound to the probe in the presence of manganese, even when Fur was present at one-third the concentration of SmcR (Fig. 9B, lane 5). In vitro competition footprinting analysis with purified Fur and SmcR showed that in a manganese-rich buffer, Fur preferentially bound to the vvsA promoter and prevented the binding of SmcR. SmcR bound to the vvsA promoter only when manganese was not sufficient or EDTA was present (Fig. 9C).

To examine further the role of SmcR in vvsA transcription, we measured transcription of vvsA::luxAB under iron-limiting...
and iron-rich conditions in wild-type and ΔsmcR strains. Under iron-rich conditions, vvsA expression was highly repressed without no significant difference between the wild-type and ΔsmcR strains, whereas under iron-limiting condition, vvsA expression is negatively regulated by SmcR at stationary phase (Fig. 10A). The same result was obtained when we measured siderophore production in wild-type and ΔsmcR strains under iron-limiting and iron-rich conditions (Fig. 10B). In the presence of iron, siderophore production was low in both wild-type and ΔsmcR strains, whereas in iron-limiting conditions, siderophore production was repressed by SmcR at stationary phase.

In summary, under iron-limiting condition, apo-Fur cannot repress the expression of vvsAB, but SmcR-mediated quorum-sensing is a main regulatory factor. Therefore, the transcription of vvsAB is derepressed at low cell density, but it is repressed by SmcR at high density. However, under iron-rich condition, Fur is the major regulatory factor repressing vvsAB expression, regardless of cell density (Fig. 11).

**DISCUSSION**

How bacteria regulate siderophore biosynthesis in response to the bacterial population remained to be elucidated.
a few studies suggesting involvement of quorum sensing in siderophore production in some bacteria. In *Burkholderia cepacia*, a mutation in *cepR*, which is a homolog of *V. harveyi luxR*, results in increased siderophore production (30). In *V. harveyi*, the *luxOD47E* mutation, which locks the bacteria in a low cell density quorum-sensing mode (23), leads to accumulation of high levels of siderophores (31). In *Pseudomonas putida* and *aeruginosa*, addition of a quorum-sensing antagonist inhibits siderophore biosynthesis (32). However, to the best of our knowledge, the molecular mechanism underlying quorum-sensing based regulation of siderophore biosynthesis has not previously been delineated. In this study, we showed that SmcR represses the transcription of the *vvs* genes by directly binding to the promoter region. The SmcR binding site (5′-AATGATAATGATAATTATT-3′) in the *vvsA* promoter is a mirror image of the known SmcR binding consensus sequence (5′-TTATTTGTWWRWWNTNAATAA-3′) (18). The *vvsA* site has a 22-bp inverted repeated sequence just like the consensus sequence, suggesting that the size and the inverted repeat feature may be more important for SmcR binding than the base pairs per se.

Site-directed mutagenesis of the *vvsA* SmcR binding site abolished the differences in the expression of the *vvsA-luxAB* fusion between ΔluxOΔsmcR and ΔluxO strains, confirming that the site is responsible for the SmcR-mediated regulation. Expression levels of *vvsA-luxAB* from the construct with the mutated SmcR binding site were significantly higher than with the wild-type promoter sequence (Fig. 6B). We suggest that the mutations also interfered with the binding of Fur to the region so that expression was fully derepressed.

The competition gel shift assay and DNaseI footprinting experiments indicate that in iron-rich media, the transcription of the siderophore synthesis (*vvsAB*) genes is repressed by the Fur-iron complex, whereas SmcR does not play a significant role. When bacteria are grown under iron-limited conditions, quorum sensing regulates siderophore production, and, at high
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cell densities, SmcR is the main regulator of transcription of vsaAB. It is well known that high intracellular iron concentrations produce reactive radicals that are toxic to cells (2). There are ~2500 Fur molecules in a cell during exponential growth, and the number increases during late log phase and stationary phase in *V. cholerae* (10). The relatively high abundance and constitutive expression of Fur in bacteria may contribute to tight regulation of iron uptake and prevent the intracellular iron concentration from reaching a harmful level.

The simplest rationale for the involvement of quorum sensing in the regulation of siderophore synthesis is to allow the pathogen to manage energy economically at high cell density. At low cell density, production of siderophores to acquire iron may be helpful to support the growth of cells. At high cell density, cell division is slowed and not as much siderophore, and iron are required. Instead, cells may utilize energy for other useful functions such as biofilm formation, which protects the pathogen from host immune attack and acts as a reservoir from which cells are released for pathogen propagation (15). Because Fur cannot function without bound iron, bacteria may need to utilize an alternative mechanism to repress the expression of siderophores in iron-limiting conditions. In this sense, quorum-sensing regulation appears to be a good alternative strategy to control intracellular iron when cells are at high cell density.

We observed that growth of a *V. vulnificus* strain with a smcR deletion is significantly slower than wild-type in iron-limited media (data not shown), suggesting that quorum-sensing regulation is beneficial for bacterial growth in iron-limiting conditions. Whether this difference in growth rate is due to siderophore synthesis needs to be explored because numerous functions are modulated by quorum sensing. For instance, smcR might regulate *ryhB* expression, which negatively regulates iron-containing proteins such as ubiquinol-cytochrome *c* reductase and superoxide dismutase in *V. cholerae* (33), and this may facilitate bacterial survival under iron-limited conditions. The smcR-mediated quorum sensing globally regulates other important functions such as oxidative stress responses and other functions for survival and propagation. Most studies of quorum sensing have been performed in iron-rich medium, which eliminates iron stress conditions, and hence the role of smcR in iron stress responses remains to be examined.

Orchestrated regulation of siderophore production by two global regulators, Fur and SmcR, ensures that cells can establish appropriate concentrations of intracellular iron to optimize survival and propagation in the human body. As described above, *V. vulnificus* harbors other functions such as homologs of vibB, vibE, and vibH in *V. cholerae* related to iron acquisition. Future studies will examine whether those functions are subject to, or modulate regulation by Fur and SmcR-mediated quorum sensing.

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