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Original Article

# SmcR, the Quorum-sensing Master Regulator, Is Partially Involved in Temperature/Salinity-mediated Changes in Metalloprotease *vvpE* Expression in *Vibrio vulnificus*

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*Vibrio vulnificus*, a gram-negative halophilic marine bacterium and opportunistic human pathogen, must withstand various environmental changes, especially simultaneous changes in temperature and salinity, from 25℃/2.5% to 37℃/0.9% (SCTS) upon entering the human body. In our previous study, SCTS stimulated *vvpE* expression even in the background of a mutation in *luxS* encoding LuxS enzyme for the biosynthesis of quorum-sensing (QS) signal molecule autoinducer-2 (AI-2), suggesting that the A1-2-mediated QS system is partially involved in the SCTS-mediated change of *vvpE* expression. In this study, we examined the effects of the QS master regulator SmcR on SCTS-mediated changes in *vvpE* expression and extracellular VvpE production. SCTS stimulated *V. vulnificus* growth, but with no increase in maximal growth levels. The SCTS-mediated prolongation of the stationary growth phase resulted in a significant increase in growth phase-dependent *smcR* and *vvpE* expressions. A mutation in *smcR* seriously repressed *vvpE* expression, but had no significant effect on *V. vulnificus* growth. However, the *smcR* mutation only partially attenuates SCTS-mediated changes in *vvpE* expression. These results indicate that SCTS stimulates the expressions of *smcR* and *vvpE* by stimulating *V. vulnificus* growth, and that SmcR is only partially involved in SCTS-mediated changes in *vvpE* expression.

**Key Words:** *Vibrio vulnificus*, Temperature, Salinity, Metalloprotease, Autoinducer-2, Quorum-sensing

## INTRODUCTION

*Vibrio vulnificus*, a gram-negative halophilic marine bacterium, is an opportunistic pathogen that causes rapidly progressing fatal septicemia and necrotizing wound infections, preferentially in susceptible patients with hepatic diseases, hemochromatosis, heavy alcohol drinking habits, and other immunocompromised conditions (1).

*V. vulnificus* produces a metalloprotease known as VvpE. Purified VvpE exhibits a variety of biological activities including hemorrhagic damage, dermonecrosis, enhanced vascular permeability, and edema, and is lethal to mice (1). Although the pathogenetic role of VvpE remains enigmatic (2), *vvpE* expression or VvpE production is a useful tool for studying how *V. vulnificus* senses and responds to diverse environmental signals, since it is the best known phenotype directly controlled by three global regulators, RpoS (the stationary sigma factor responsible for general stress response), SmcR (the master regulator of quorum sensing [QS] systems responsible for bacterial density-dependent gene expression), and Crp (cAMP-receptor pro-

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tein responsible for catabolite repression) (3, 4). In addition, extracellular VvpE production occurs via the type II general secretion system (GSS), the activity of which is influenced by environmental factors (5~7).

The precise role of QS is still a matter of debate. Nevertheless, QS probably plays a vital role in environmental response and gives each bacterium a survival advantage (8). *V. vulnificus* possesses two QS systems. First, autoinducer (AI)-2 signaling is finally transferred to the master regulator SmcR (a LuxR homolog) via LuxP, LuxQ, LuxU, LuxO, and LuxT (9). SmcR regulates *vvpE* expression at the transcription level by directly binding to the *vvpE* promoter in *V. vulnificus* (3). Second, another QS signal molecule acylated homoserine lactone (AHL: AI-1) was recently identified in *V. vulnificus*, although no known AHL-related gene was detected (10). AHL signaling is also likely to be finally transferred to SmcR in *V. vulnificus*, as in *V. harveyi* (11). The presence of the two QS systems implies that they can cooperate, and that *smcR* mutation that blocks both AI-2 signaling and AHL signaling is more suitable for the complete disruption of QS than *luxS* mutation, which blocks only AI-2 signaling.

*V. vulnificus* inhabits sea water and can infect the human body, and these two environments differ in many respects. As a result, *V. vulnificus* must overcome and survive diverse environmental changes to establish successful human infections. Virulence expression is a survival strategy of pathogenic bacteria (12), and *V. vulnificus* is capable of sensing and responding to diverse environmental changes by modulating the expressions of the virulence factors required for survival (13, 14). Many environmental factors including temperature, salinity, iron, and oxygen levels can affect *vvpE* expression (15). In particular, a temperature of 25℃ and a salinity of 2.5% mimics estuarine condition in the summer, and 37℃ and 0.9% the human body condition. Previous studies have suggested that *V. vulnificus* might sense and respond to temperature and salinity changes by modulating *vvpE* expression via AI-2-QS system (15, 16). In our previous study (17), a simultaneous change of temperature and salinity from 25℃/2.5% to 37℃/0.9% (SCTS) stimulated *vvpE* expression even in the background of a mutation in *luxS* encoding LuxS enzyme, which is required for AI-2 biosynthesis (18). These findings suggest that the A1-2-QS system is only partially involved in the SCTS-mediated modulation of *vvpE* expression. This obscurity regarding the role played by the AI-2-QS system may be because the *luxS* mutation disrupts only AI-2 signaling, and this may result in an increase in AHL signaling. Accordingly, in an attempt at obtaining unequivocal evidence regarding the involvements of the QS systems, we examined the effect of a *smcR* mutation that can simultaneously block AI-2 and AHL signaling, on the SCTS-mediated modulation of *vvpE* expression and extracellular VvpE production under the same condition as in our previous study (17).

## MATERIALS AND METHODS

### Bacterial strains, media, and reagents

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Heart Infusion (HI; Becton-Dickinson (BD), Franklin Lakes, NJ, USA) broth and agar containing 2.5% sodium chloride were used to cultivate *V. vulnificus* strains. Luria-Bertani medium (BD) and Thiosulfate-Citrate-Bile-Sucrose medium (BD) with or without appropriate antibiotics were used to select and subculture recombinant strains. Antibiotics (BD) were used at the following concentrations; for *Escherichia coli*, ampicillin 50 μg/ml, kanamycin 50 μg/ml, tetracycline 12.5 μg/ml, and chloramphenicol 30 μg/ml; for *V. vulnificus*, ampicillin 20 μg/ml, kanamycin 200 μg/ml, tetracycline 2 μg/ml, and chloramphenicol 2 μg/ml. Unless otherwise stated, all reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Culture condition, bacterial growth measurement, and β-galactosidase assay

*V. vulnificus* strains were preconditioned by culturing in HI broth containing 2.5% NaCl at 25℃ overnight to adapt them to conditions mimicking their natural habitat. The preconditioned strains were inoculated into HI broth containing 0.9% NaCl or 2.5% NaCl at $1 \times 10^6$ cells/ml and cultured with vigorous shaking (220 rpm) at 37℃ or 25℃

**Table 1.** Bacterial strains, plasmids and primers used in this study

| Strains, plasmids and primers | Relative characteristics and sequences | References |
|---|---|---|
| *V. vulnificus* | | |
| M06-24/O | Highly virulent clinical isolate | 19 |
| CMM2101 | [a]M06-24/O with a $lacZ_{Vv}$ mutation | 18 |
| RC174 | M06-24/O with a *smcR* mutation | This study |
| RC186 | RC174 with an *in trans smcR* complementation | This study |
| RC196 | [a]CMM2101 with a merozygotic $P_{smcR}$::$lacZ_{Ec}$ transcriptional fusion | This study |
| CMM2106 | CMM2101 with a $P_{vpE}$::$lacZ_{Ec}$ transcriptional fusion | 18 |
| RC164 | CMM2106 with a *smcR* mutation | 4 |
| *E. coli* | | |
| SY327 λ*pir* | Host for suicide vector | 20 |
| SM10 λ*pir* | Conjugation donor | 20 |
| Plasmid | | |
| pDM4 | Suicide vector with R6K origin; [b]$Cm^R$ | 21 |
| pQF52 | IncP *lacZ* transcriptional fusion vector; [b]$Amp^R$ | 22 |
| pLAF3 | IncP cosmid vector; [b]$Tc^R$ | 23 |
| pLAF3II | pLAFR3 with *bla* inserted at the cos site; $Amp^RTc^R$ | 18 |
| pRK2013 | IncP, [b]$Km^R$, Tra $Rk2^+$ *repRK2 repE1* | 24 |
| pUTKm1 | Tn5-based insertion delivery plasmid, $Amp^R$ | 25 |
| pRC152 | pDM4 with a 2.05-kb *Sma*I-*Spe*I fragment containing the in-frame deleted *smcR* gene | This study |
| pRC158 | pLAFR3II with a 1.39-kb *Hin*dIII-*Eco*RI fragment containing the *smcR* gene | This study |
| pRC162 | pQF52 with a 926-bp *Bam*HI-*Hin*dIII fragment containing the *smcR* regulatory region | This study |
| pRC164 | pDM4 with a *Bam*HI-*Sca*I fragment containing the $P_{smcR}$::*lacZ* fragment from pRC162 | This study |
| Primers | | |
| smcR-up-1 | [c]5'-tcc**CCCGGG**ttatttatctcaacctttgcccac-3' | This study |
| smcR-up-2 | 5'-ttactggtgttacatgagctgctgtttacgttttag-3' | This study |
| smcR-down-1 | 5'-cagcagctcatgtaacaccagtaacctcatatcaag-3' | This study |
| smcR-down-2 | [c]5'-g**ACTAGT**gaaagctgctgatcatgggtgg-3' | This study |
| smcR-comp-1 | [c]5'-ccc**AAGCTT**gttcaatggcacgagcaagatcc-3' | This study |
| smcR-comp-2 | [c]5'-g**GAATTC**tgttggtctagcggcggaagtg-3' | This study |
| smcR-rep-F | [c]5'-cg**GGATCC**caaagccaatccacttcactgg-3' | This study |
| smcR-rep-R | [c]5'-ccc**AAGCTT**gccacgacgagcaaacacttcc-3' | This study |

[a] *Vv* and *Ec* stand for *V. vulnificus* and *E. coli*, respectively.
[b] $Cm^R$, $Amp^R$, $Tc^R$, and $Km^R$ stand for chloramphenicol-, ampicillin-, tetracycline-, and kanamycin-resistance, respectively.
[c] Capital bold letters indicate the restriction enzyme-recognition sequences: CCCGGG for *Sma*I, ACTAGT for *Spe*I, AAGCTT for *Hin*dIII, GAATTC for *Eco*RI, and GGATCC for *Bam*HI.

for 24 h. Culture aliquots were withdrawn at appropriate times to measure bacterial growth and β-galactosidase activity. To measure extracellular VvpE production, culture supernatants were obtained from culture aliquots by cen-

trifugation (10,000 × *g* for 5 min). Bacterial growth was measured by determining the optical densities of culture aliquots at a wavelength of 600 nm ($OD_{600}$). β-Galactosidase activity in culture aliquots was measured using the Miller method (26).

**Western blotting for VvpE**

Rabbit polyclonal anti-VvpE antibody was prepared and VvpE production was measured using Western blotting, as described in our previous study (5). In brief, equal volumes (20 μl) of culture supernatants were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were incubated with rabbit polyclonal anti-VvpE antibody and subsequently with anti-rabbit-IgG antibody conjugated with alkaline phosphatase. Finally, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium solution was used for visualization. The amount of VvpE on Western blot was quantitatively analyzed using a densitometer program (UN-SCAN-IT gel Automatid Digitalizing System ver. 4.1, Silk Scientific Corporation).

**Mutation and complementation of *smcR***

*V. vulnificus* M06-24/O, a highly virulent clinical isolate (19), was used as the wild-type strain. A deletion mutant in *smcR* was constructed using the suicide vector pDM4 with a R6K origin (21). The 5' and 3' ends of *smcR* were amplified by polymerase-chain reaction (PCR) using the primers; smcR-up-1/2 and smcR-down-1/2. The resulting PCR products were used as the DNA template for the second crossover PCR using the primers; smcR-up-1 with a *Sma*I overhang and smcR-down-2 with a *Spe*I overhang. The resulting *Sma*I-*Spe*I fragment with deleted *smcR* was cloned into pDM4. The resulting plasmid was named pRC152, and transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (20), and subsequently transferred to M06-24/O by conjugation. Finally, a stable transconjugant was selected on TCBS agar containing chloramphenicol and on HI agar containing 10% sucrose, and named RC174. The deletion mutation of *smcR* was confirmed by PCR using the primers smcR-up-1 and smcR-down-2.

To restore wild-type *smcR* in RC174 with mutated *smcR*, the *Hin*dIII-*Eco*RI fragment containing the promoter region and the open reading frame of *smcR* was amplified using the PCR primers; smcR-comp-1 with a *Hin*dIII overhang and smcR-comp-2 with a *Eco*RI overhang, and was subsequently subcloned into the broad host range vector pLAFR3II (18), which was prepared by subcloning the *Bam*HI-*Bgl*II fragment containing the *bla* gene of pUTKm1 into a *Bgl*II site of pLAFR3 (24, 25). The resulting plasmid was named pRC158 and transferred into RC174 by triparental mating using the conjugative helper plasmid pRK2013 (24). Finally, a stable transconjugant was selected on TCBS agar containing ampicillin and tetracycline and named RC186. The presence of wild-type *smcR* on plasmid DNA was confirmed by PCR using the primers smcR-comp-1 and 2.

**Construction of *lacZ*-fused transcription reporter strains**

*V. vulnificus* CMM2101 with a *lacZ* mutation (18), which was originated from M06-24/O, was used as the wild-type strain for the construction of transcriptional reporter strains. *V. vulnificus* CMM2106 with the $P_{vvpE}$::*lacZ* transcription fusion was constructed as described previously (18). *V. vulnificus* RC164 was constructed by introducing a *smcR* mutation into *V. vulnificus* CMM2106 with the $P_{vvpE}$::*lacZ* transcription fusion, as described above. The merozygotic $P_{smcR}$::*lacZ* transcription reporter was constructed as follows. A 926-bp *Bam*HI-*Hin*dIII fragment containing the promoter region of *smcR* was amplified using the PCR primers smcR-rep-F with *Bam*HI overhang and smcR-rep-R with *Hin*dIII overhang, and the resulting PCR product was subcloned into pQF52 (22). From the resulting plasmid pRC162, the *Bam*HI-*Sca*I fragment containing the $P_{smcR}$::*lacZ* fragment was isolated and subcloned into pDM4 (21). The resulting plasmid pRC164 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (20), and transferred to CMM2101 by conjugation. Eventually, a transconjugant (RC196) was selected on TCBS agar containing chloramphenicol. The presence of the $P_{smcR}$::*lacZ* fusion in RC196 was confirmed by β-galactosidase assay (26).

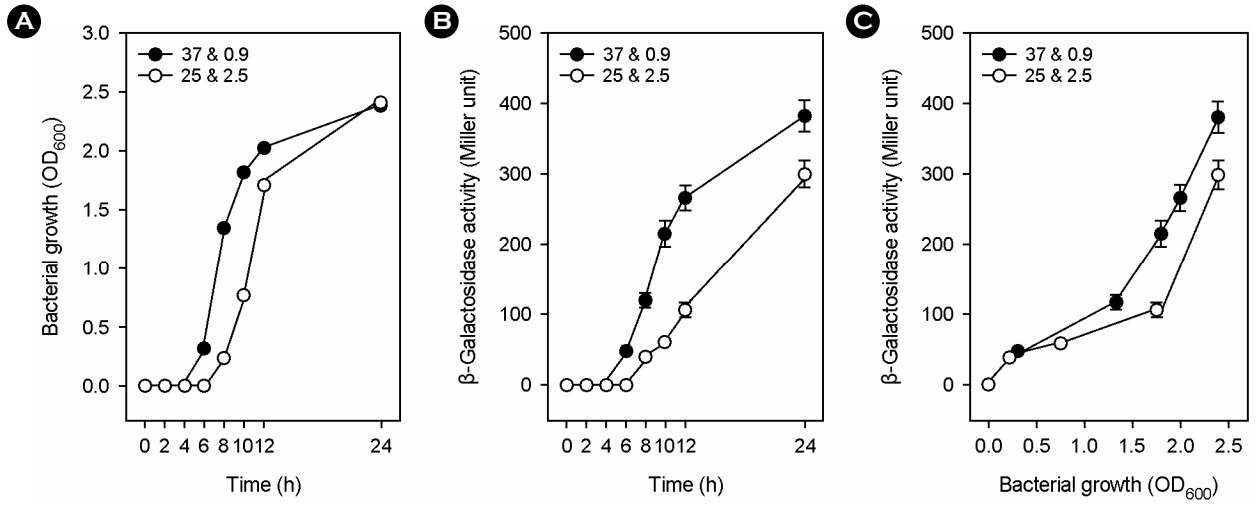


**Figure 1.** Effect of a temperature and salinity change from 25℃/2.5% to 37℃/0.9% on *smcR* expression. After preconditioning by culturing at 25℃/2.5% overnight, the *V. vulnificus* RC196 strain with the $P_{smcR}$::*lacZ* transcriptional fusion was transferred into fresh broths and cultured at 25℃/2.5% or 37℃/0.9%. Bacterial growth (A) is expressed as the optical densities of culture aliquots at 600 nm ($OD_{600}$). Accumulated β-galactosidase activity in culture aliquots is expressed in Miller units, and plotted against culture time (B) and bacterial growth (C). Means and standard deviations are of triplicate measurements.

## RESULTS

The effect of SCTS on *smcR* expression was determined using RC196 with the merozygotic $P_{smcR}$::*lacZ* transcriptional fusion. SCTS stimulated and advanced RC196 growth with no significant difference in the maximal growth level, and subsequently prolonged the duration of the stationary growth phase in 24 h-batch culture (Fig. 1A). The pattern of *smcR* expression was growth phase- and bacterial density-dependent (Fig. 1B and 1C): *smcR* expression was initiated at low bacterial densities, gradually increased during the exponential growth phase, and thereafter steeply increased at high bacterial densities or during the stationary growth phase. SCTS significantly increased *smcR* expression levels on a per-cell basis. Overall, these results indicate that SCTS can increase total QS activity by facilitating *smcR* expression and *V. vulnificus* growth.

The effects of SCTS on *vvpE* expression in the background of wild-type or mutated *smcR* were determined using the two $P_{vvpE}$::*lacZ* transcriptional reporter strains, CMM2106 with wild-type *smcR* and RC164 with mutated *smcR*. SCTS stimulated and advanced the growths of the two strains, and prolonged the duration of the stationary growth phase (Fig. 2A and 2D). No significant difference in the growth levels of the two strains was observed, indicating that SmcR does not affect *V. vulnificus* growth. In CMM2106, the pattern of *vvpE* expression was growth phase- and bacterial density-dependent (Fig. 2B and 2C): *vvpE* expression was initiated at low bacterial densities, gradually increased during the exponential growth phase, and thereafter steeply increased at high bacterial densities or during the stationary growth phase. SCTS did not increase *vvpE* expression levels on a per cell basis during the exponential growth phase. However, SCTS significantly increased *vvpE* expression levels once the growth of CMM-2106 arrived at the stationary growth phase. Accordingly, SCTS increased total *vvpE* expression levels, and prolonged the duration of the stationary growth phase in 24-h batch culture. In RC164, *vvpE* expression was severely repressed, and the pattern of *vvpE* expression was not growth phase-dependent: *vvpE* expression increased during the exponential growth phase, but not during the stationary growth phase. Nevertheless, SCTS also increased *vvpE* expression levels in RC164. However, when compared quantitatively, the SCTS-mediated increase in *vvpE* expression was approximately 4-fold in CMM2106 and approximately 2-fold in RC164. These results indicate that SCTS facilitates growth

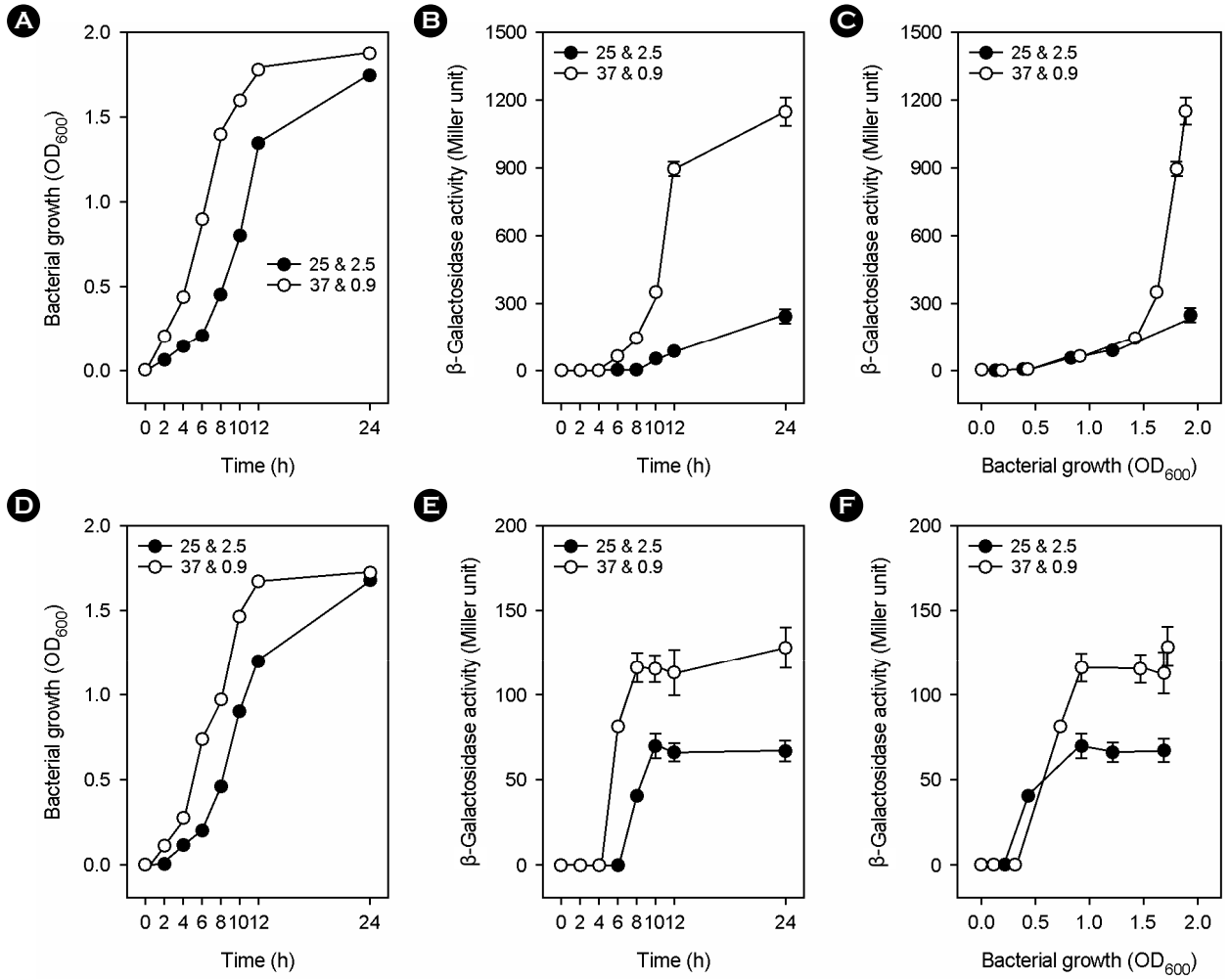


**Figure 2.** Effect of *smcR* mutation on the regulation of *vvpE* expression induced by a change of temperature and salinity from 25℃/2.5% to 37℃/0.9%. After preconditioning by culturing at 25℃/2.5% overnight, the two *V. vulnificus* strains with $P_{vvpE}$::*lacZ* transcriptional fusion, CMM2106 with wild-type *smcR* (A to C) and RC164 with mutated *smcR* (D to F) strains, were transferred to fresh broths and cultured at 25℃/2.5% or 37℃/0.9%. Bacterial growth (A and D) is expressed as optical densities of culture aliquots at 600 nm ($OD_{600}$). Accumulated β-galactosidase activity in culture aliquots is expressed in Miller units, and plotted against culture time (B and E) and bacterial growth (C and F). Means and standard deviations are of triplicate measurements.

phase-dependent *vvpE* expression by stimulating *V. vulnificus* growth, and that SmcR partially affects SCTS-mediated changes in *vvpE* expression.

The effects of SCTS on extracellular VvpE production in the background of wild-type *smcR*, mutated *smcR*, and *in trans* complemented *smcR* were determined using the three strains, M06-24/O with wild-type *smcR*, RC174 with mutated *smcR*, and RC186 with *in trans* complemented *smcR*. SCTS stimulated and advanced the growth of all three strains, and prolonged the duration of the stationary growth phase (Fig. 3A). The *smcR* mutation or *in trans* complementation had no significant effect on *V. vulnificus* growth. In M06-24/O, SCTS advanced and stimulated extracellular VvpE production, and this was consistent with growth levels (Fig. 3B). SCTS or the *smcR* mutation did not affect the pattern of extracellular VvpE production: 35 kDa-VvpE was the main form under all conditions. At 25℃/2.5%, extracellular VvpE production was first observed at 10~11 h after culture initiation, and at 37℃/0.9%, extracellular VvpE production was first observed at 4 or 5 h after culture initiation. The *smcR* mutation in RC174 lowered and delayed extracellular VvpE production, and these changes

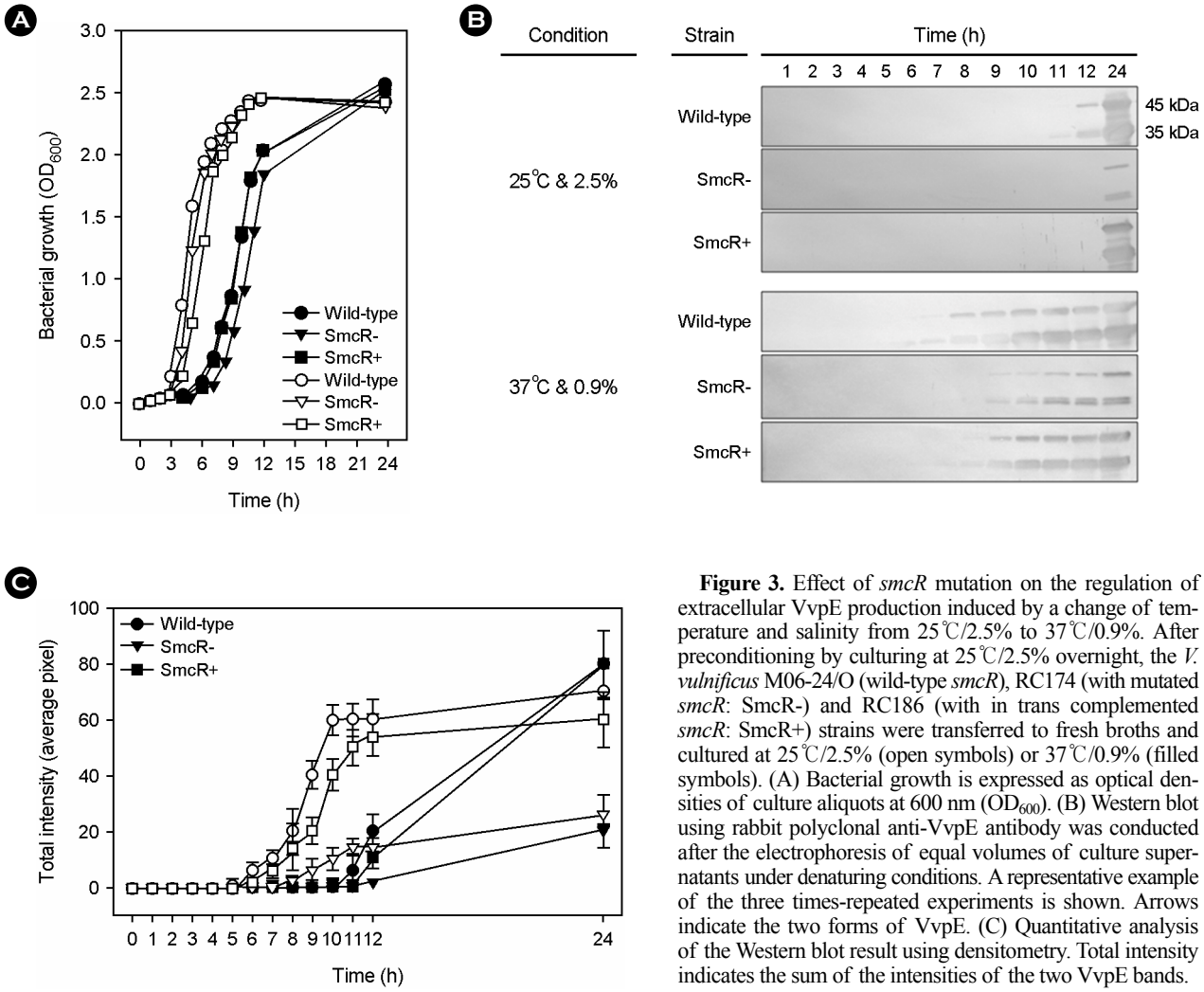


**Figure 3.** Effect of *smcR* mutation on the regulation of extracellular VvpE production induced by a change of temperature and salinity from 25℃/2.5% to 37℃/0.9%. After preconditioning by culturing at 25℃/2.5% overnight, the *V. vulnificus* M06-24/O (wild-type *smcR*), RC174 (with mutated *smcR*: SmcR-) and RC186 (with in trans complemented *smcR*: SmcR+) strains were transferred to fresh broths and cultured at 25℃/2.5% (open symbols) or 37℃/0.9% (filled symbols). (A) Bacterial growth is expressed as optical densities of culture aliquots at 600 nm ($OD_{600}$). (B) Western blot using rabbit polyclonal anti-VvpE antibody was conducted after the electrophoresis of equal volumes of culture supernatants under denaturing conditions. A representative example of the three times-repeated experiments is shown. Arrows indicate the two forms of VvpE. (C) Quantitative analysis of the Western blot result using densitometry. Total intensity indicates the sum of the intensities of the two VvpE bands.

were recovered by the *in trans smcR* complementation in RC186. The SCTS-mediated change in extracellular VvpE production appeared to be slightly attenuated but was still observed in RC174 with the *smcR* mutation: extracellular VvpE production was observed from 12 h at 25℃/2.5% and from 8 h at 37℃/0.9%. When quantitative analyzed using densitometry (Fig. 3C), the SCTS-mediated change in extracellular VvpE production was also still observed in RC174 with the *smcR* mutation. These results indicate that SCTS facilitates extracellular VvpE production, stimulating *V. vulnificus* growth, and that SmcR is not the main factor responsible for SCTS-mediated changes in extracellular VvpE production.

## DISCUSSION

The present study shows that SCTS stimulates the expressions of *smcR* and *vvpE* by stimulating *V. vulnificus* growth, and that SmcR is only partially involved in SCTS-mediated changes in *vvpE* expression.

Virulence expression is a survival strategy of pathogenic bacteria (8). *V. vulnificus* inhabits sea water and can infect the human body. During the infection process, *V. vulnificus* must be able to sense and sustain changes in environmental factors including SCTS (13~17). Signals emanating from changes in environmental factors are relayed to specific genes by way of cognate signal transduction systems and

result in the expressions of genes associated with specific virulence factors. Our previous (17) and present studies show that SCTS stimulates *V. vulnificus* growth and facilitates *vvpE* expression or extracellular VvpE production. These findings suggest that VvpE functions as one of the several virulence factors required for the better survival of *V. vulnificus* on entering the human body. As there have been serious doubts concerning the pathogenetic role of VvpE (2), a new paradigm is required to elucidate the currently obscure pathogenetic roles of VvpE. Generally, bacteria grow asynchronously, for example, *V. vulnificus* cells at different phases of growth can exist simultaneously in cultures, and this is invariably the case *in vivo*. Furthermore, the VvpE produced by *V. vulnificus* cells that have arrived at the stationary growth phase can affect the growth or survival of *V. vulnificus* cells beginning to grow, by destroying various host proteins. In our previous study, we found that VvpE can destroy various host proteins, such as lactoferrin and immunoglobulin A and that VvpE is essentially required for *V. vulnificus* swarming (27). Bacterial swarming is defined as a rapid and coordinated population migration of flagellated bacteria across solid surfaces, and is considered a good model of adherence to, colonization on, and invasiveness into mucosa, which are essential steps during early infection (28). Moreover, the destruction of the iron-withholding protein lactoferrin and immunoglobulin A, both of which are important components for mucosal immunity, by VvpE may affect the growth and survival of *V. vulnificus* at the early stage of infection (29). Accordingly, VvpE is likely to play significant roles in adherence to, colonization on, and invasiveness into mucosa by *V. vulnificus* during early infection, although VvpE is mainly produced at the stationary growth phase *in vitro*.

It is believed that QS plays a vital role in environmental response, and that it provides a survival advantage, although its precise role is still a matter of debate (8). A mutation in *luxS* or *smcR* lowers virulence expression in experimental mouse models (18, 30), and QS actually operates in a human *ex vivo* experimental system (submitted). In our previous study (17), SCTS stimulated and advanced *V. vulnificus* growth with no increase in the maximal growth level, and the consequent prolongation of the stationary growth phase significantly increased growth phase-dependent *luxS* expression. In the present study, SCTS significantly increased *smcR* expression. These findings indicate that the activity of QS systems is higher at 37℃/0.9% than at 25℃/2.5%. The expression level of *luxS* alone cannot represent actual AI-2-QSS activity or *smcR* expression level because AI-2 (furanosyl borate diester) production is mediated by several spontaneous non-enzymatic reactions in addition to the enzymatic action of LuxS (31). Moreover, another QS signal molecule AHL may also affect *smcR* expression (10). AI-2 signaling is almost completely transferred to SmcR, and AHL signaling is considered to be finally transferred to SmcR in *V. vulnificus*, and the two QS systems can cooperate to improve survival in *V. vulnificus*, as in *V. harveyi* (11). Accordingly, the expression level of *smcR* is likely to directly represent the total or final activity of the QS systems. The *smcR* mutation adopted in this study is more suitable for the complete disruption of QS than the *luxS* mutation adopted in our previous study (17). In addition, QS activity should be evaluated with *vvpE* expression, because *vvpE* expression is the best known target directly regulated by SmcR. In this study, the *smcR* mutation seriously repressed *vvpE* expression, which reconfirms that SmcR, the master regulator of QS systems, regulates *vvpE* expression.

Temperature-dependent expression of virulence genes has been extensively studied in *Shigella* species (32, 33). *Shigella* infection is spread via the fecal-oral route, and is commonly acquired by drinking water contaminated with human fecal material. Virulence genes in *Shigella* species, including *ipa* (invasion plasmid antigen), *spa* (surface presentation antigens) and *mxi* (membrane expression of invasion) operons, are induced at 37℃ and repressed at 30℃. Changes in DNA supercoiling and the transcription repressor H-NS (VirR) are likely to play important roles in the temperature-dependent regulation of virulence genes. VirF positively regulates *virB* transcription, and VirB acts as a positive regulator for the transcriptions of the *ipa*, *mxi*, and *spa* operons. An increase in temperature activates the VirF-dependent *virB* promoter by changing DNA supercoiling. In addition, *virB* and *virF* transcriptions are

repressed by H-NS (VirR) at a low temperature, but derepressed at a permissive temperature (i.e., 37℃). Although there is no experimental evidence, similar mechanisms may also be involved in the temperature-dependent expressions of *smcR* and *vvpE* in *V. vulnificus*.

In our previous study (17), the SCTS-mediated modulations of *vvpE* expression and extracellular VvpE production were slight attenuated in the background of *luxS* mutation. In the present study, the SCTS-mediated modulations of *vvpE* expression and extracellular VvpE production were also slight attenuated but was still observed in the background of *smcR* mutation. Taken together, these findings indicate that the QS systems are only partially involved in the SCTS-mediated changes of *vvpE* expression and extracellular VvpE production. In addition to SmcR or QS signals, another factor is likely to be involved in the SCTS-mediated change of *vvpE* expression. We found that SCTS prolonged the duration of the stationary growth phase by stimulating *V. vulnificus* growth, and significantly increased the expression level of *vvpE* only during the prolonged stationary growth phase in 24-h batch culture. These findings indicate that the duration of the stationary growth phase plays an important role in the stimulation of growth phase-dependent *vvpE* expression. SCTS-mediated growth stimulation and the resulting prolongation of the stationary growth phase, may increase metabolic or oxidative stress, and result in Crp and/or RpoS increases. Furthermore, Crp and SmcR synergistically affect RpoS-dependent *vvpE* promoter (3). Our recent research also revealed that Crp functions as an essential activator, and SmcR functions as a modulator for full *vvpE* expression only in the presence of Crp (4). It has been reported that Crp can affect *rpoS* expression in *V. vulnificus* (34). We also recently found that Crp can affect *smcR* expression in *V. vulnificus* (submitted). Accordingly, Crp alone or in combination with SmcR may be involved in the SCTS-mediated change of RpoS-dependent *vvpE* expression.

In addition to *vvpE* expression, SCTS also appeared to facilitate extracellular VvpE production. In a previous study (5), we found that two forms of VvpE are secreted via type II GSS after being produced within bacterial cells. These suggest that SCTS may also affect the activity of type II GSS. In another study, we found that iron can facilitate the extracellular secretion of cytolysin/hemolysin VvhA by increasing the activity of type II GSS (7). Accordingly, it has been suggested that iron can increase both *vvpE* expression and extracellular VvpE production (35).

In summary, SCTS was found to facilitate both *smcR* and *vvpE* expression by stimulating *V. vulnificus* growth. However, SCTS-mediated changes in *vvpE* expression were found to be slightly attenuated in the background of *smcR* mutation. Accordingly, it is likely that SCTS stimulates the expressions of *smcR* and *vvpE* by stimulating *V. vulnificus* growth, and that SmcR is only partially involved in SCTS-mediated changes in *vvpE* expression.

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